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SCHOOL OF PUBLIC HEALTH

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PRICES SUBJECT TO CHANGE

ENVIRONMENTAL MICROBIOLOGY

AS RELATED TO PLANETARY QUARANTINE

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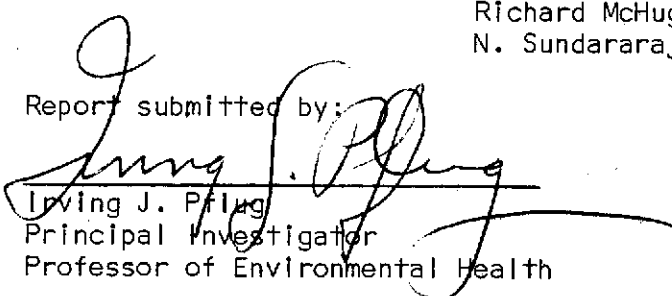

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INTRODUCTION

This report covers research activities during the period June 1, 1972 through November 30, 1972 for the project entitled "Environmental Microbiology as Related to Planetary Quarantine." These studies were conducted by the Division of Environmental Health, School of Public Health, at the University of Minnesota under the auspices of Dr. Lawrence B. Hall, Planetary Quarantine Officer, National Aeronautics and Space Administration. These studies were carried out in the Environmental Sterilization Laboratory that is part of the Space Science Center, University of Minnesota.

This is the ninth semi-annual report of progress on NASA project NGL 24-005-160. A limited number of our earlier reports on this project are still available and can be obtained by requesting copies.

In this report, the experimental design for the study to evaluate the effect of different cleaning methods and storage conditions on the dry heat resistance of Bacillus subtilis var. niger spores are described and the results for the first evaluation are reported. We anticipate that this study will be continued until the end of this overall NGL 24-005-160 which will probably give us at least three years of observation on the effect of these different conditions on the survival and heat resistance of Bacillus subtilis var. niger spores.

In section 3D of this report, we are redefining the intercept ratio so that the initial numbers of microorganisms will not have an effect on the intercept ratio (IR value) that is obtained. We believe that this is a definite improvement in establishing a second parameter for identifying the survivor curve.

Sterilization microbiology requires a microbiological, an engineering and a statistical or biometrical input. Faculty members in the Division of Biometry, School of Public Health, University of Minnesota and graduate students in this division have been associated with this project throughout its life. They provide direct input in the design of experiments and in the evaluation of results. They have assisted and in some cases designed the computer programs for evaluating the data. Their association with our project has stimulated them to carry out basic research in these areas of microbial destruction. Dr. R. B. McHugh, Professor of Biometry and

Dr. N. Sundararaj who completed his doctorate under Dr. McHugh's direction have carried out studies on the decimal reduction time (D-value) estimation in thermal microbial sterilization. A preliminary report of their work is included in Appendix D of this report. Additional information on these studies can be obtained from the thesis entitled, "The Biometric Analysis of Non-linear Models with Applications to Disinfection" by N. Sundararaj[1] which is available on microfilm.

I. J. Pflug

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[1]N. Sundararaj, "The Biometric Analysis of Non-linear Models with Applications to Disinfection," Ph.D. Thesis University of Minnesota, 1971.

I. EFFECT OF COMBINED HEAT AND RADIATION ON MICROBIAL DESTRUCTION

Donald A. Fisher and Irving J. Pflug

INTRODUCTION

This is the continuation of a study that was initiated in June 1971.
Prior to this reporting period a prototype controlled environmental system
for use in this study was designed, constructed and tested. The University
of Minnesota radiation source was mapped. (See Progress Reports #7 and #8.)

During the past six months we have studied the destruction of bacterial
spores subjected to both wet heat and combined wet heat and low level gamma
radiation. Evidence has been found that different radiation induced microbial
destruction mechanisms are in effect at the low and at the high temperatures
studied. The effect of the mechanism operative over the high temperature
range resembles the synergistic effect reported by Sandia Laboratories of
Albuquerque, New Mexico.

OBJECTIVES

Objectives of this project are to investigate the synergistic effect
which occurs when spores are subjected simultaneously to dry heat and gamma
radiation so as to be able to specify thermoradiation sterilization cycles
and at the same time, develop a better understanding of the underlying
mechanism(s) that lead to spore death from this combination of stresses.
To accomplish these objectives we have: (I) investigated the survival of
spores on surfaces at various temperatures in a precisely controlled
environmental system, (2) determined the rate of destruction of these spores
at ambient temperature when subjected to gamma radiation, and (3) determined
the rate of destruction of these spores when they are subjected to combined
gamma radiation and thermal stress.

MATERIALS AND METHODS

To study the combined effect of wet heat and gamma radiation, bacterial
spores were suspended in a phosphate buffer in sealed glass test tubes. The
temperature of the suspension was controlled during the gamma radiation
treatment.

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Spore Suspension

Spores of _Bacillus subtilis_ var. _niger_ (AAHF) were suspended in 5 ml of Sorensen's phosphate buffer (pH 7.0) in screw-capped glass test tubes (13 X 100 mm). The population density of the suspension was approximately 10^6 spores per ml. After inoculation, the tubes were refrigerated at 4°C until time for testing (usually 24 to 72 hours).

Exposure Chamber

The exposure chamber shown in Figure 1.1 is an element of the controlled temperature water circulating system that was designed to hold a spore suspension test tube and to allow the test tube to be easily inserted and/or removed. The chamber is a tubular aluminum vessel in which the test tube is held rigidly while the circulating water flows around the test tube. The chamber is thermally insulated by using polyether foam. A thermocouple is located adjacent to the test tube. To minimize radiation attenuation, the walls of the chamber were made from relatively thin aluminum and the water thickness between the radiation source and sample was kept small. A photograph of a chamber is shown in Figure 1.2. A number of samples could be exposed to different levels of radiation concurrently as shown in Figure 1.3.

Temperature Control

The temperature of the spore suspensions was controlled by the temperature of the water stream that circulated in series through all exposure chambers. This stream was pumped from a constant temperature reservoir; the reservoir temperature was controlled to within $\pm$ 0.1°C by an RFL proportional controller. This controller was located outside the Gamma Irradiation Facility to prevent damage from the radiation.

Tests were conducted to determine if there were significant differences in temperature among exposure chambers. When the chambers were connected in series, there was no measureable temperature difference between the first and last chambers. When the system was operated at 90°C without radiation, the spore destruction characteristics were the same for each of the chambers. These results suggest that any difference in temperature among chambers was below the sensitivity level of the spores, our most accurate differential thermometer.

Heatup rate

Once the circulation pump was started the spore suspension took a finite time to reach the temperature of the circulating water. This heating

POLYETHER
FOAM
INSULATION

O-RING

WATER
OUTLET

GAMMA
RADIATION

BRASS SPRING
HOLDER

ALUMINUM EXPOSURE
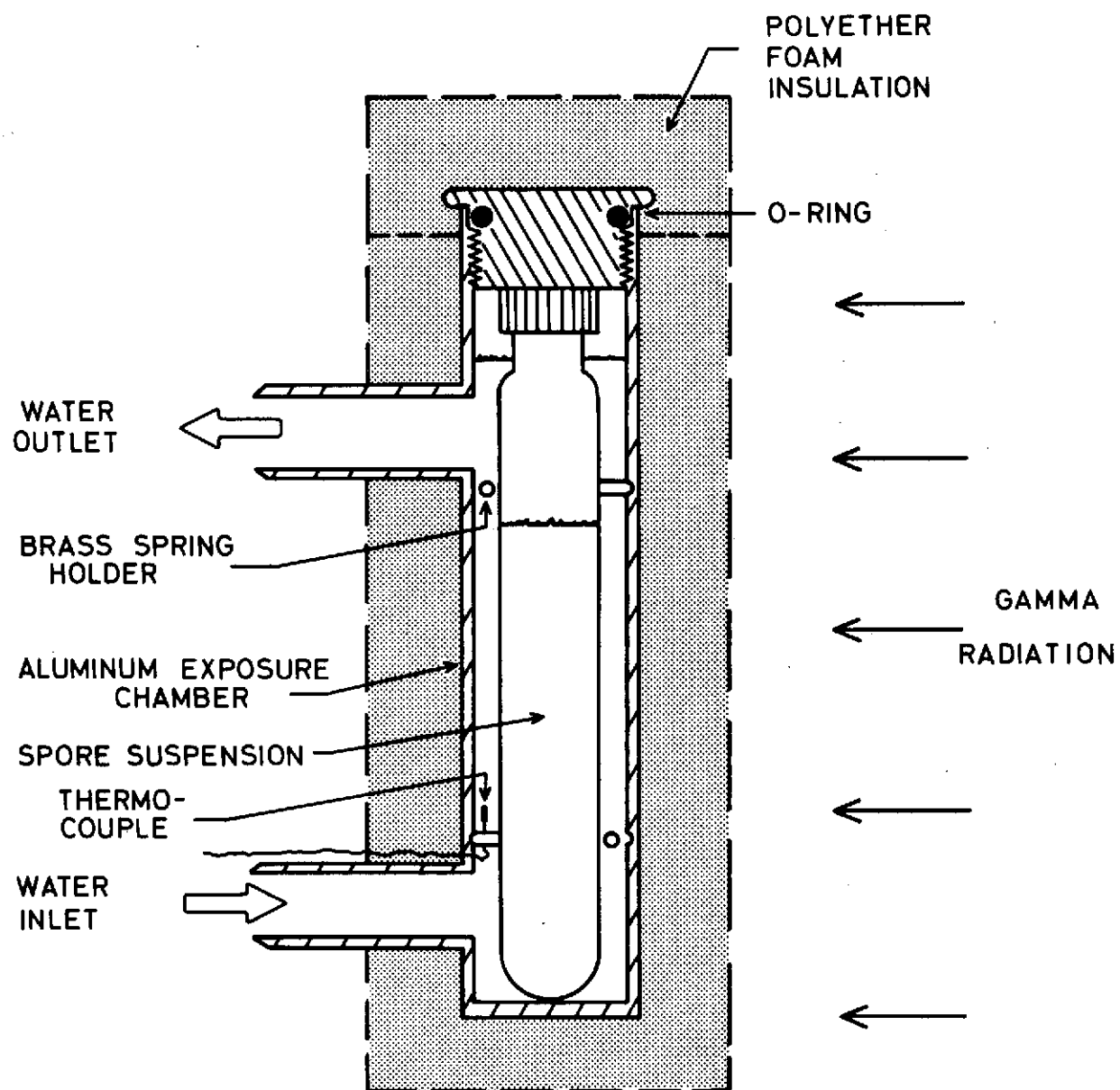CHAMBER

SPORE SUSPENSION

THERMO-
COUPLE

WATER
INLET

Figure I.I: Sectional view of exposure chamber used in wet heat
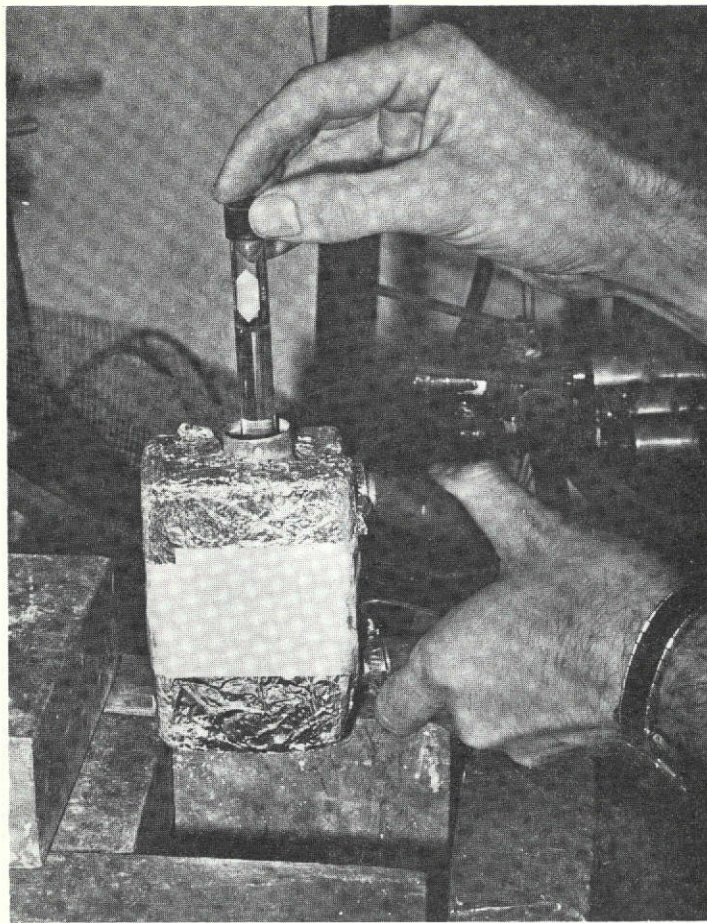thermoradiation study.

Figure I.2: Exposure chamber used in wet heat thermoradiation study.
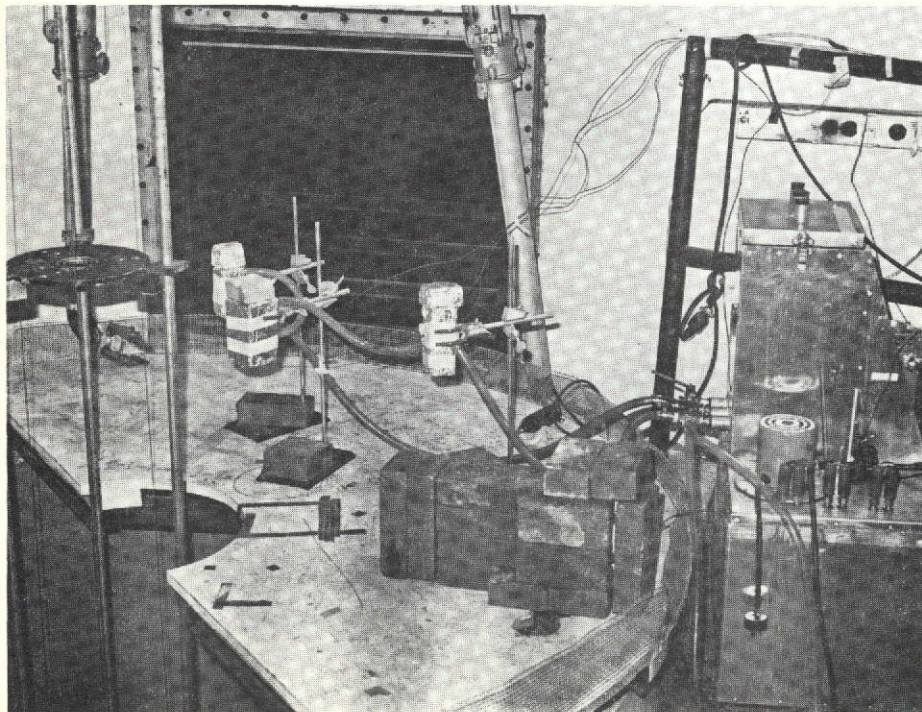

Figure I.3: Overall view of the equipment in Gamma Facility.

lag was determined by placing a thermocouple at the geometric center of
5 ml of cold water in a 13 X 100 mm test tube. The startup procedure
of the system was simulated and the rate of temperature rise was monitored.

Figure 1.4 shows the time dependency of the reduced temperature
difference $[T(t) - T_o]/[T_F - T_o]$ at the center of the tube $[T(t) =$ tempera-
ture at tube center, $T_o =$ initial temperature and $T_F =$ final temperature$]$
for two heating simulations and one cooldown simulation. From Figure 1.4
we observe that 50 seconds after the start of the water circulation system the
spore suspension has reached 99% of its total temperature change. Although
this temperature lag introduces some error into the experiment, when viewed
relative to the long exposure times used in the experiment the heatup lag
is relatively insignificant.

Radiation sources and dosimetry

The Cesium-137 radiation sources were described in Progress Report #7.
The radiation dose rate at various positions around the sources was measured
using Fricke Dosimetry as a primary reference and a calibrated Vitoreen
Rate Meter as a secondary reference. The exposure dose rates used were 20,
10, 5, and 0 Krd/hr.

Using the source, locations where desired radiation intensities occurred
were identified. The exposure chambers were then spatially located around
the radiation sources at the predetermined positions. Thus, a number of
samples could be exposed to different levels of radiation concurrently as
shown in Figure 1.3.

Heated non-radiated sample

Concurrent with the thermoradiation experiment, spores were subjected
to only thermal stress. Similar 13 X 100 mm test tubes containing 5 ml of
buffer and spores were used. These tubes were placed in an exposure chamber
identical to the ones used for the thermoradiation exposures. However,
this chamber was surrounded by at least 5 cm thickness of lead shielding,
with an extra 25 cm thickness of lead shielding between the radiation sources
and the sample. The experimental setup is shown in Figure 1.5. With the
shielding in place, the radiation level inside the lead compartment was
2.4 rd/hr or less than .05% of the level of the lowest dose rate used in
the thermoradiation experiments. The heated non-radiated samples received
a greater amount of radiation than would have occurred from background
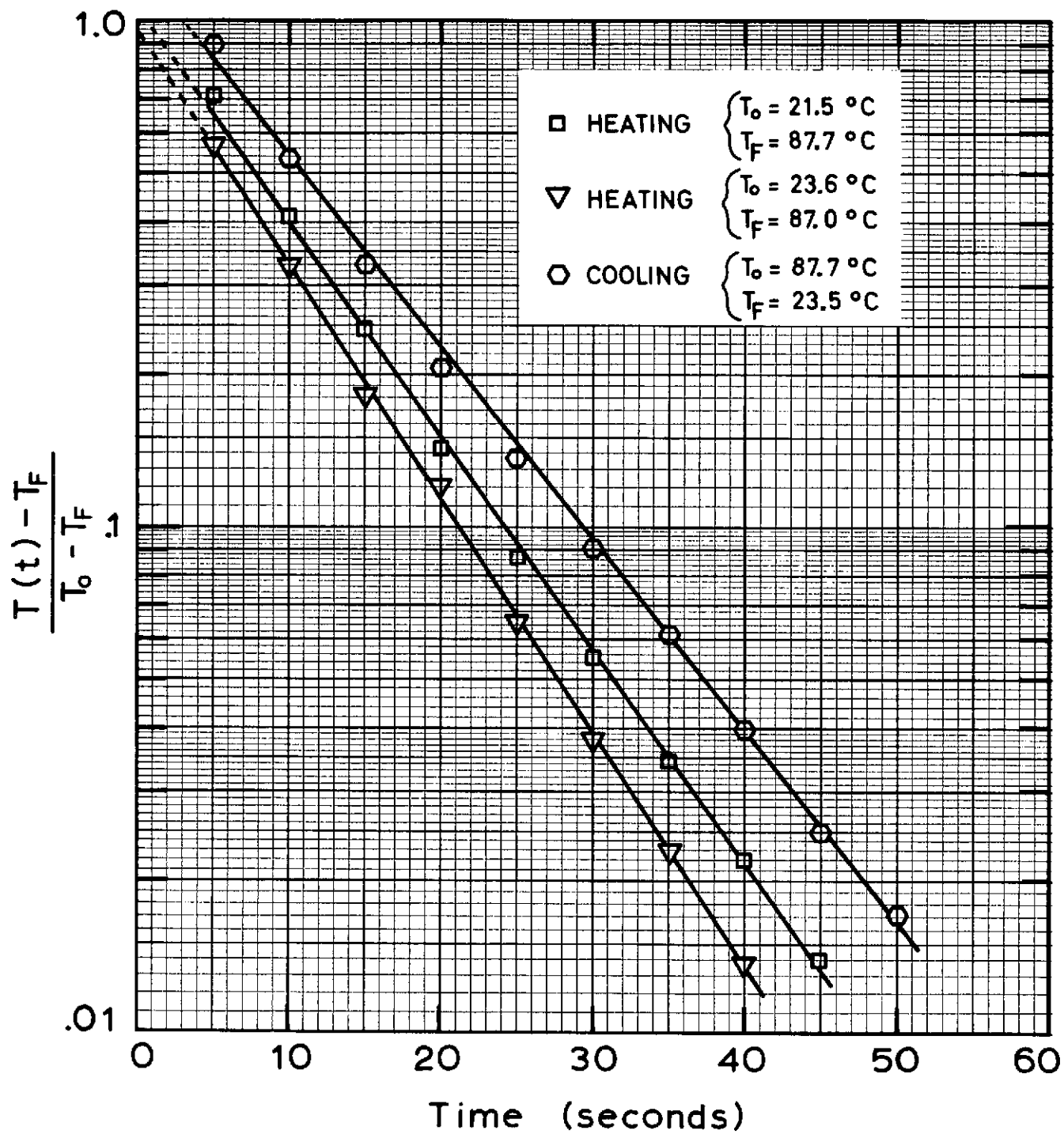
Figure 1.4: Heating and cooling curves for spore suspension in wet heat thermoradiation study.

radiation alone; however, compared to the radiated samples, they received an insignificant amount of radiation.
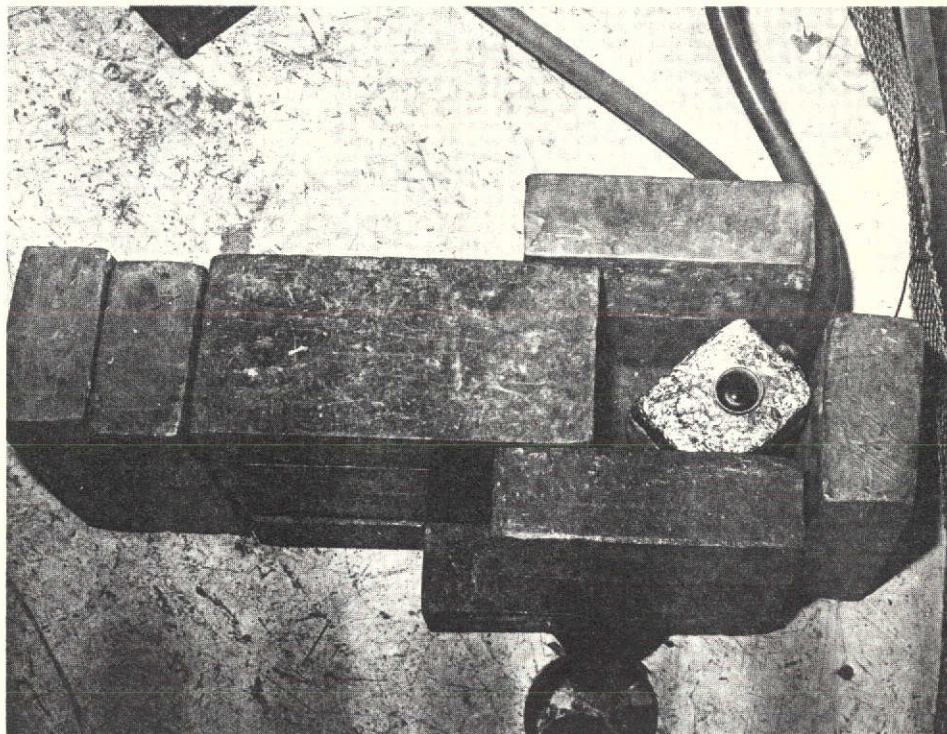
Figure I.5: Lead shielding of sample for no radiation control.

Sequence of Operations

All tests were carried out in the same general manner.   The operations were carried out in the following order:

1.   First, the temperature of the water bath was stabilized at the test temperature.

2.   The constant temperature bath was moved into the Gamma Irradiation Facility where it was connected to hoses leading to the exposure chambers.

3.   The exposure chambers were positioned at their experimental location (sites of known radiation intensities).

4.   The test tubes containing spores were then inserted into the exposure chambers and the chamber covers were secured.

5.   The experiment was set to run.

6.   The water circulation pump was turned on.   The Gamma Facility was closed and personnel safety precautions were taken.   The Cesium sources were raised from the facility pool into the operating position at which time the treatment was considered to have been started.   The time delay from start of circulating pump until positioning of the sources was approximately 40 seconds.

7.   The temperature in the sample chambers was monitored continuously during the treatment period.   The output from the thermocouple in the exposure chamber was measured using a temperature recording potentiometer (Honeywell, Model #15).   The operator observed these readings during the exposure period; adjustments were made as required in the temperature of the water bath to maintain the experiment within control limits.

8.   At the end of the exposure period, the sources were lowered into the gamma absorbing pool, safety checks were completed, the Gamma Facility was opened and the circulation pump was shut down.

9.   The samples were then quenched by circulating cold tap water through the series of chambers.

10.   After a 1 1/2 minute cooling period, the exposure chambers were opened, sample tubes removed and placed in ice water and untreated samples placed in the exposure chamber.

11.   The system was now ready for the entire procedure to be repeated for the next treatment period.

The treatment periods were successive to one another; the sequence was chosen in a random fashion.

After treatment, the sample tubes were stored in an ice bath and assayed as soon as possible.   The elapsed time from treatment to assay varied from immediately afterwards to about 15 hours.   A test was performed to determine if the elapsed time from treatment to assay had an effect on the number of surviving spores recovered.   Spore suspensions were assayed immediately after treatment, sample tubes were then refrigerated and assayed a second time after 24 hours storage.   There was no appreciable difference between the number of viable spores recovered immediately after treatment and the number recovered after storage for 24 hours.

To recover the treated spores the sample tube was placed on a vortex mixer.   A one ml aliquot was pipetted from the tube, diluted in buffered distilled water and plated in duplicate using Trypticase soy agar.   Plates were incubated at 32°C for 48 hours.

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RESULTS

The results of the several experiments are summarized in Table I.I.
Survivor curves are shown in Figures I.6 through I.I2. Each figure
contains the results for a single temperature; there are separate survivor
curves for each radiation level studied.

Table I.I

D-values (hours) at various temperatures and exposure rate for wet-heat
thermoradiation of Bacillus subtilis var. niger (AAHF)

Test Number	Temperature °C	D-values (hours)			
		Dose rate (Krd/hr)			
		20	10	5	0
DF2291A-D	22	7.01	13.92	28.34	1141.[a]
DF2312P-S	45	7.627	17.06	37.78	-851.[a]
DF2303A-D	60	11.62	15.65	29.46	2564.[a]
DF2300A-D	75	6.09	8.79	16.02	67.48[b]
DF2298A-D	80	2.34	2.82	3.66	6.07
DF2286E-H	85	1.02	1.32	1.57	2.08
DF2286A-D	90	.352	.406	.440	.498

[a] Essentially infinite for time span of experiments

[b] Estimate (insufficient data for accurate analysis)

FIGURE 1.6: SURVIVOR CURVES FOR WET HEAT THERMORADIATION OF AAHF AT 14°C.

FIGURE 1.7: SURVIVOR CURVES FOR WET HEAT THERMORADIATION OF AAHF AT 45°C.

FIGURE 1.8: SURVIVOR CURVES FOR WET HEAT THERMORADIATION OF AAHF AT 60°C.

FIGURE 1.9: SURVIVOR CURVES FOR WET HEAT THERMORADIATION OF AAHF AT 75°C.

FIGURE 1.10: SURVIVOR CURVES FOR WET HEAT THERMORADIATION OF AAHF AT 80°C.

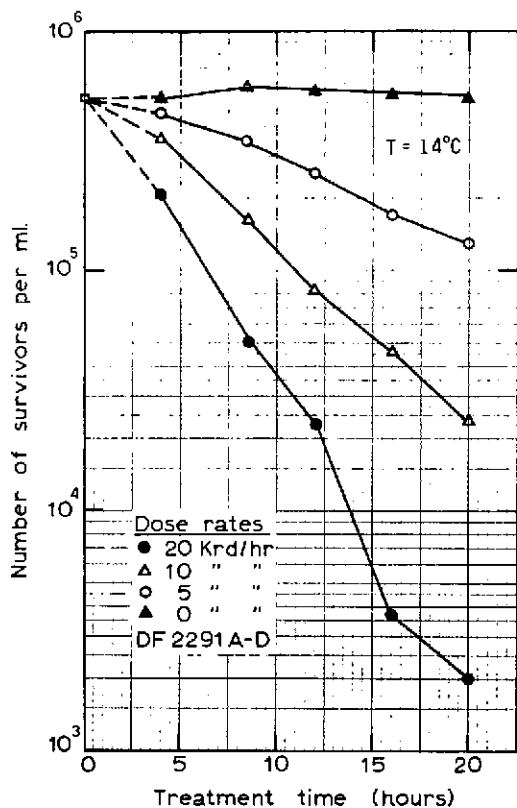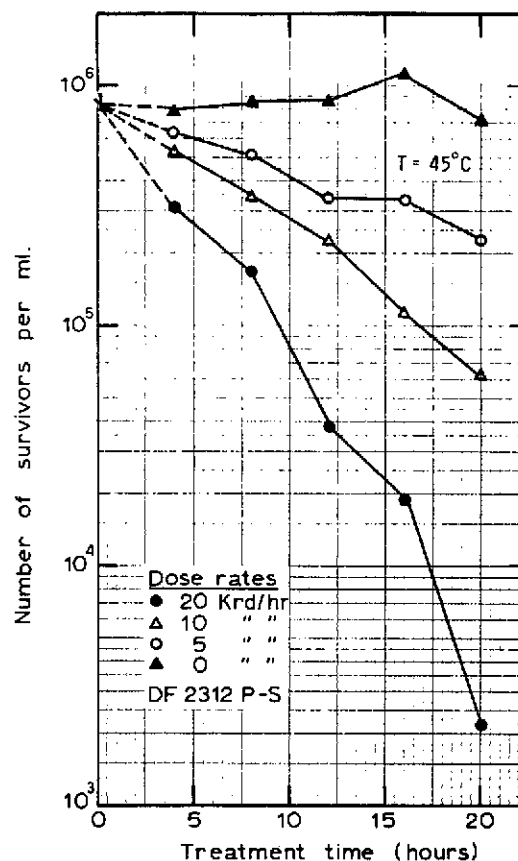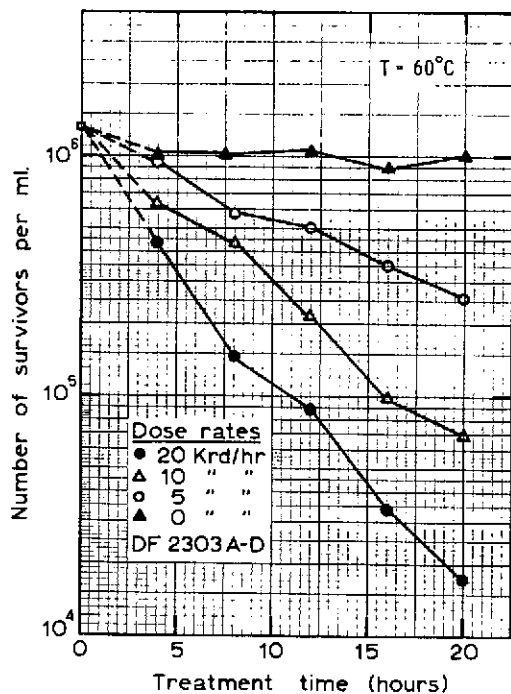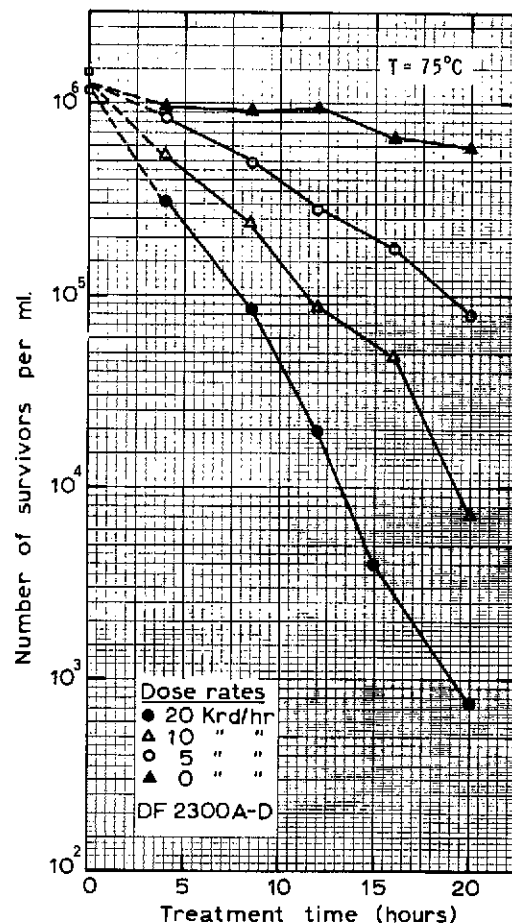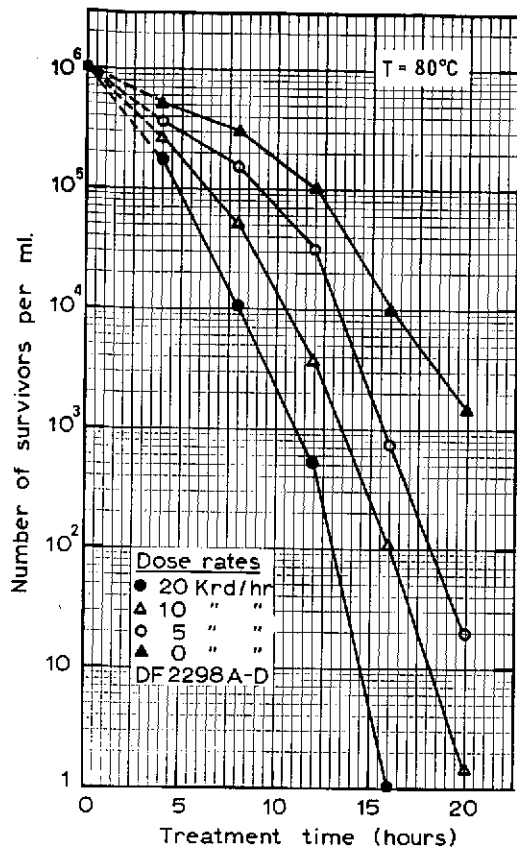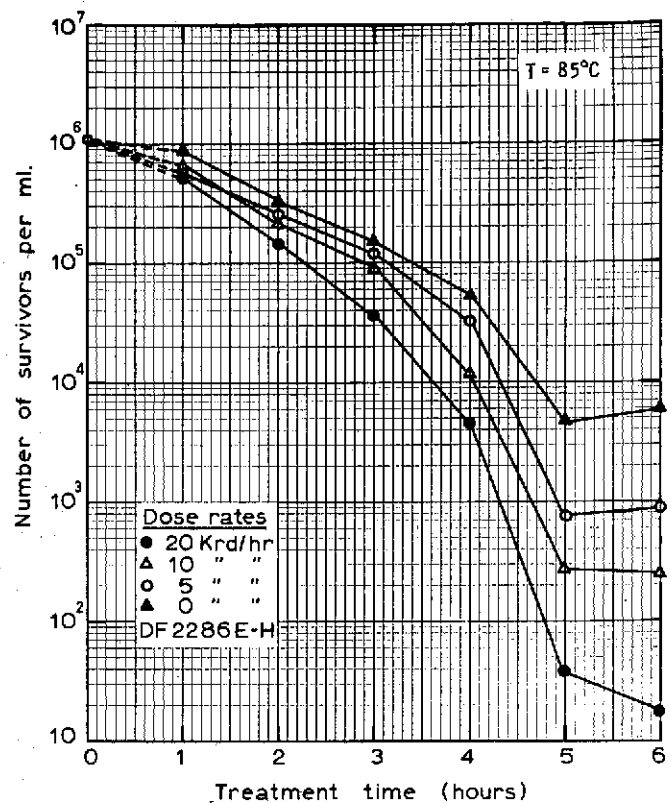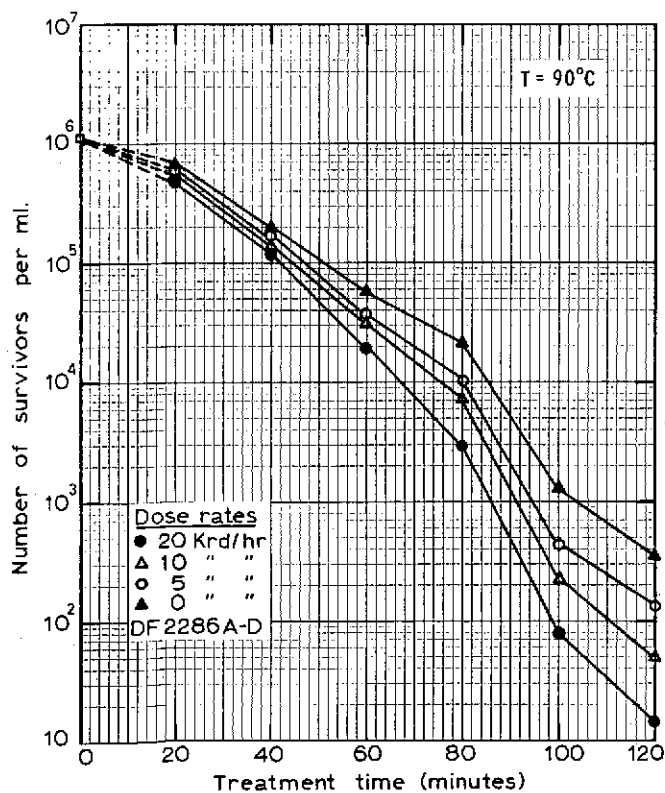
FIGURE 1.11: SURVIVOR CURVES FOR WET HEAT THERMORADIATION OF AAHF AT 85°C.

FIGURE 1.12: SURVIVOR CURVES FOR WET HEAT THERMORADIATION OF AAHF AT 90°C.

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Rationale for Experiment

The reasons for performing wet heat thermoradiation studies have a
widespread basis. First of all, from an experimental standpoint, wet
heat provides an experimental situation which is more easily controlled
than dry heat and therefore yields more accurate measurements of lethal
conditions.

Secondly, from a convenience standpoint, experimental times generally
are shorter for wet heat than for dry heat at the same temperature and
can therefore be performed more expediently.

Thirdly, from a scientific viewpoint, in a wet heat study, the exact
water activity is known inside the spore and we are not left with the
uncertainty that the radiation somehow affects the sorbed water level,
thereby augmenting the death rate.

Lastly, from an application standpoint it is interesting to know if
a synergistic mechanism is present for wet heat, bearing in mind applications
in the food, pharmaceutical, and sewage treatment industries.

Data analysis

Although the survival data does not follow exact linear survival
relationships in all cases, least squares analysis of the survivor data
can be performed for each combination of thermal and radiation stresses.
The radiation contribution at a given temperature could therefore be
approximated by taking the difference between the reciprocal D-value of the
respective radiation and no radiation curves; however, using this method
we are forcing data which is obviously in part non-linear into a linearized
form and trying to ascribe differences in the respective linearized forms
to different radiation levels. An alternate method is as follows: at
the most general level, a thermoradiation sterilization process can be
schematically represented as

$$L \;\nearrow^{\;k_T}\; E$$
$$\;\searrow_{\;k_R}\; E$$
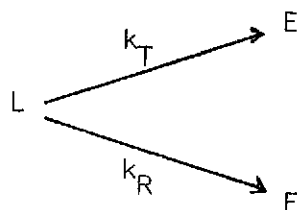
where L represents a live spore

E represents a dead spore

k_T is the kinetic parameter attributed to thermal destruction processes

k_R is the kinetic parameter attributable to all remaining death mechanisms which is absent if radiation was not present.

Thus, the resulting differential equation which would describe rate of deaths is

$$\frac{d \ N_{TR}(t)}{dt} = (-k_T - k_R) \ N_{TR}(t) \qquad (1.1)$$

and $\qquad N_{TR}(t) = N_0$ at $t = 0$.

where N_{TR} is the number of viable organisms after thermoradiation treatment for period t. Integration of Eq.(1.1) yields

$$\ell n \ [\ \frac{N_{TR}(t)}{N_0} \ ] = \int_0^t k_T dt - \int_0^t k_R dt \qquad (1.2)$$

(If k_T and k_R are independent of time this equation reduces to the familiar linear log N vs t relationship.)

If there is no radiation present, the number of survivors N_T will have the following dependency:

$$\ell n \ [\frac{N_T(t)}{N_0}] = - \int_0^t k_T dt \qquad (1.3)$$

Combining equations 1.2 and 1.3, the radiation effect can be isolated as

$$\ell n \ [\ \frac{N_{TR}(t)}{N_T(t)} \ ] = - \int_0^t k_R dt \qquad (1.4)$$

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Thus experimental values for $N_{TR}(t)$ and $N_T(t)$ can be used to quantify the radiation effect. Plots of this ratio are given in Figures 1.13 through 1.19 for each temperature.

This method of analysis provides certain advantages since we do not need to assume that the parameters k_T and k_R are constant in time in order to isolate the radiation contribution to death of a spore population. The disadvantage of this method is that estimates of N_T and N_{TR} are both subject to experimental variation and statistical noise factors. The quotient of these two estimates magnifies this noise factor somewhat, expecially if their values are close to one another.

Nevertheless, the ratio does give us results which appear to be logarithmic in nature and can be subjected to a least squares analysis. Figure 1.20 shows the logarithm of the effective D-value attributed to radiation-induced death versus temperature on a recriprocal ° K scale. Plotted in this manner, the graph would be a straight line if only one mechanism were present with an Arrhenius temperature dependency. In reality, the curves remain approximately horizontal over the lower range of temperatures but break sharply downward over the higher temperature range. This experimental behavior is generally considered to be indicative of different mechanisms being dominant over the different temperature ranges.

The curves in Figure 1.20 corresponding to different radiation levels are similar in shape. The vertical distance between any two adjacent curves remains approximately equal to log (2) over the range of temperatures. This consequence indicates that the pre-exponential coefficient of the kinetic constant(s) is(are) proportional to the radiation dose rate. In fact, if the D_R-values are expressed in terms of total exposure and plotted in the Arrhenius fashion as shown in Figure 1.21, the results generally are reduced to a single curve.

The slope of the curves over the range of 75°C to 90°C is a measure of the energy of activation for the dominant mechanism over this range. A least-squares analysis can be performed on these data, the results of which are listed in Table 1.2. Also listed in Table 1.2 is the energy of activation calculated from experiments where heat is the only lethal agent. Figure 1.22 shows the Arrhenius plots from which these results were calculated.

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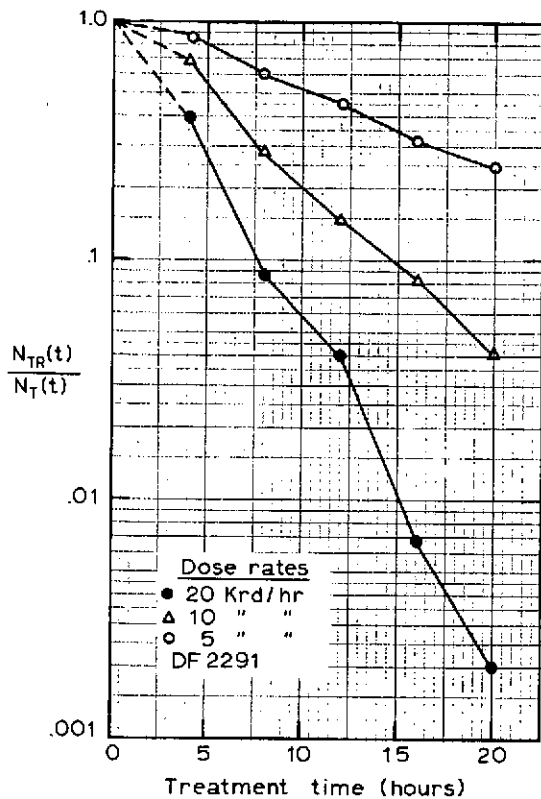
FIGURE 1.13: RATIO OF NUMBER OF SURVIVORS USING THERMORADIATION TO THE NUMBER OF SURVIVORS USING HEAT ONLY AT 14°C.
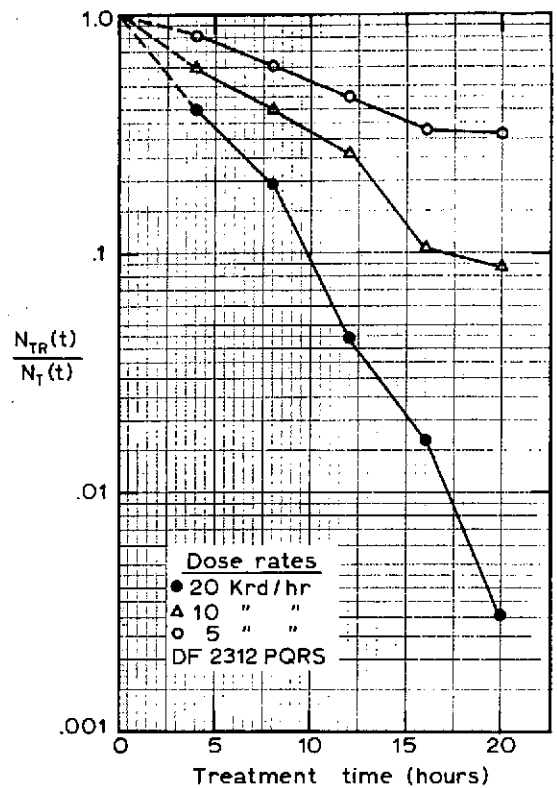
FIGURE 1.14: RATIO OF NUMBER OF SURVIVORS USING THERMORADIATION TO THE NUMBER OF SURVIVORS USING HEAT ONLY AT 45°C.
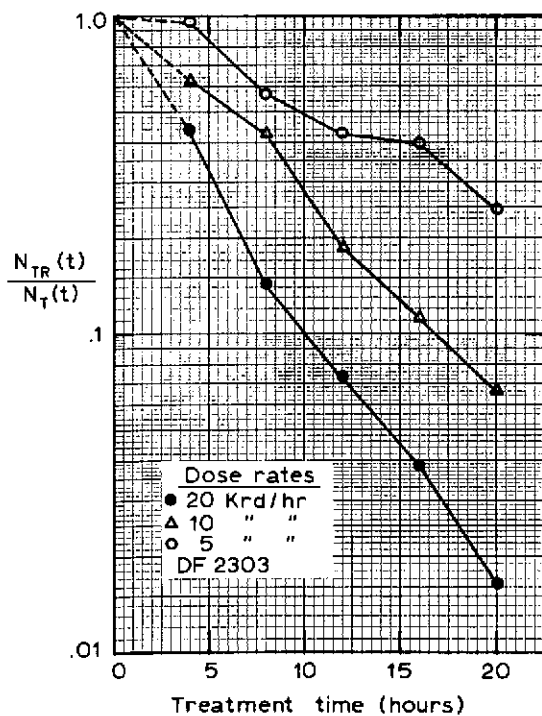
FIGURE 1.15: RATIO OF NUMBER OF SURVIVORS USING THERMORADIATION TO THE NUMBER OF SURVIVORS USING HEAT ONLY AT 60°C.
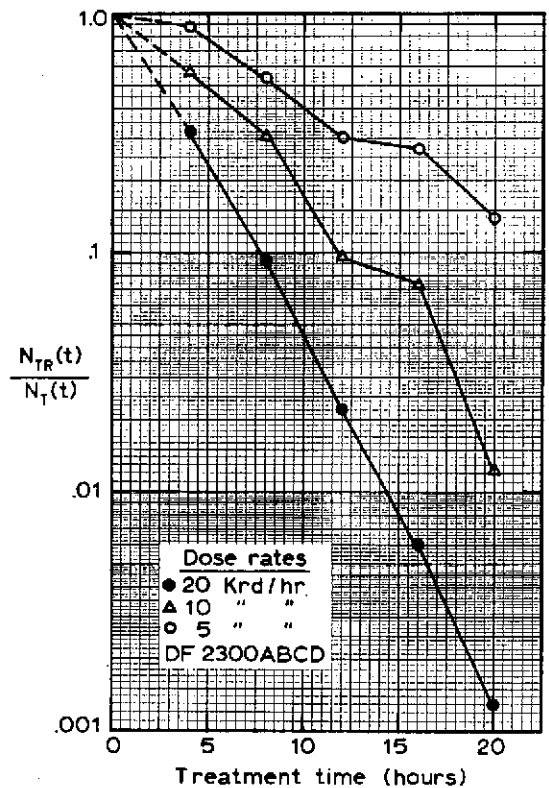
FIGURE 1.16: RATIO OF NUMBER OF SURVIVORS USING THERMORADIATION TO THE NUMBER OF SURVIVORS USING HEAT ONLY AT 75°C.
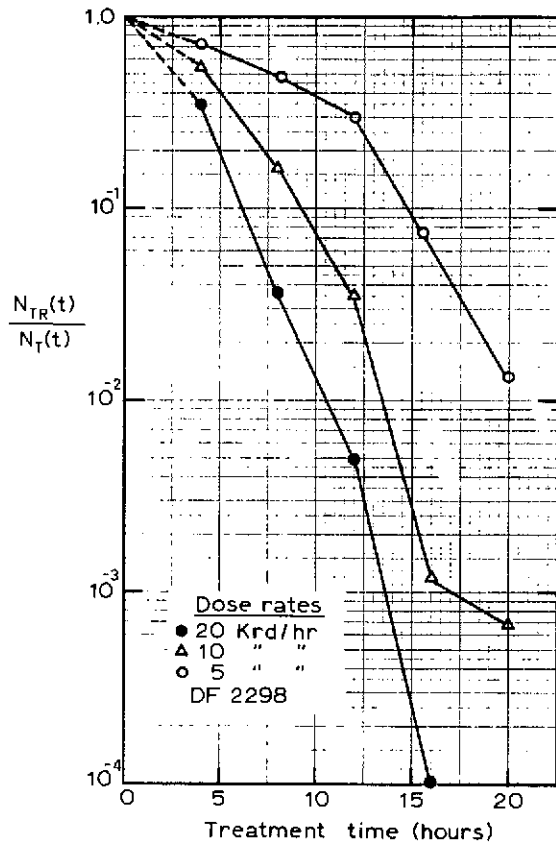
FIGURE I.17: RATIO OF NUMBER OF SURVIVORS USING THERMORADIATION TO THE
NUMBER OF SURVIVORS USING HEAT ONLY AT 80°C.
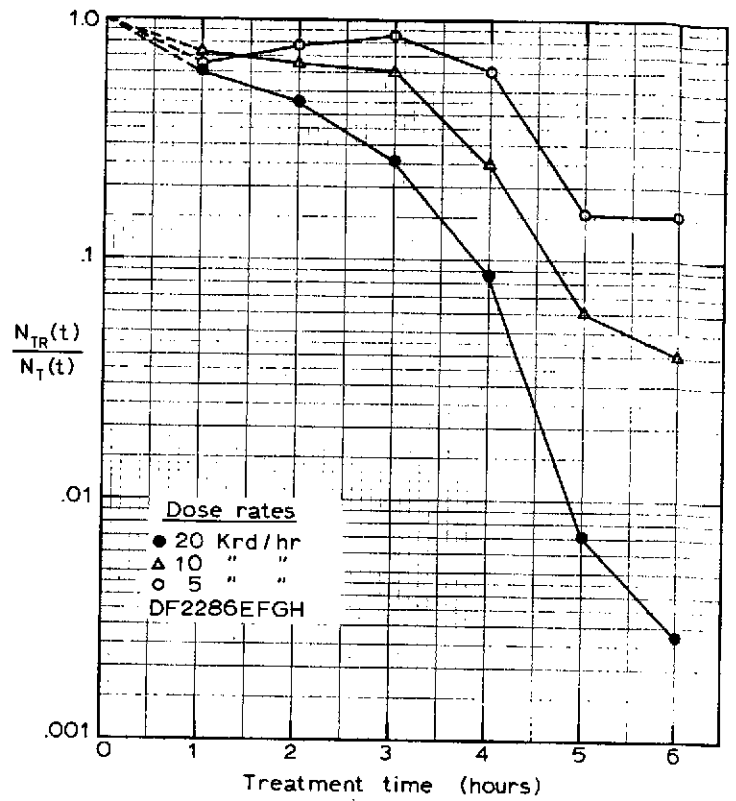
FIGURE I.18: RATIO OF NUMBER OF SURVIVORS USING THERMORADIATION TO THE
NUMBER OF SURVIVORS USING HEAT ONLY AT 85°C.
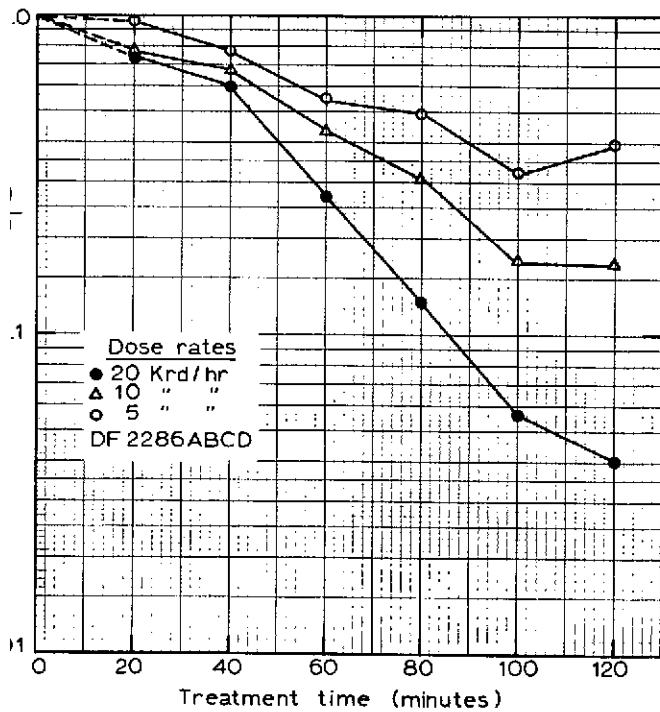
FIGURE I.19: RATIO OF NUMBER OF SURVIVORS USING THERMORADIATION TO THE
NUMBER OF SURVIVORS USING HEAT ONLY AT 90°C.

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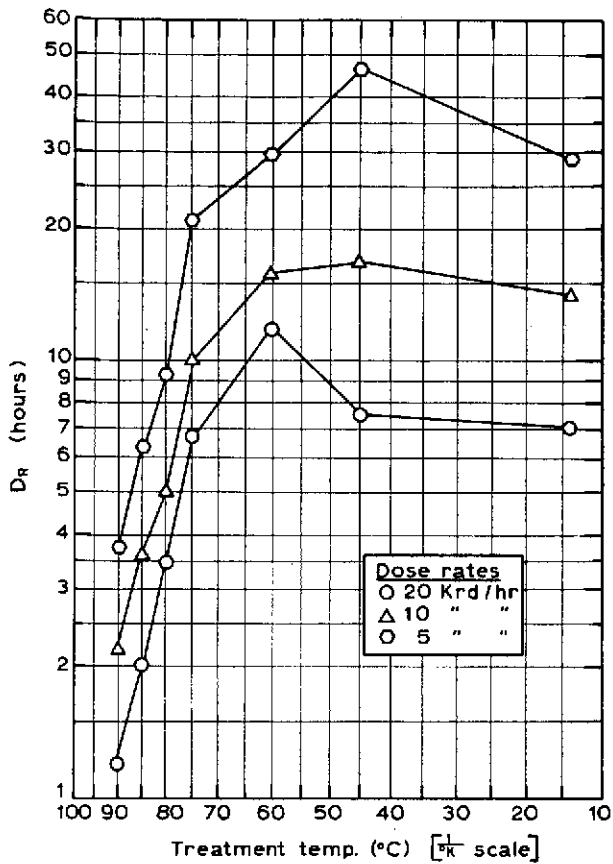
FIGURE 1.20: ARRHENIUS PLOT OF THE D-VALUE (HOURS) FOR RADIATION INDUCED DEATH FOR WET HEAT THERMORADIATION.
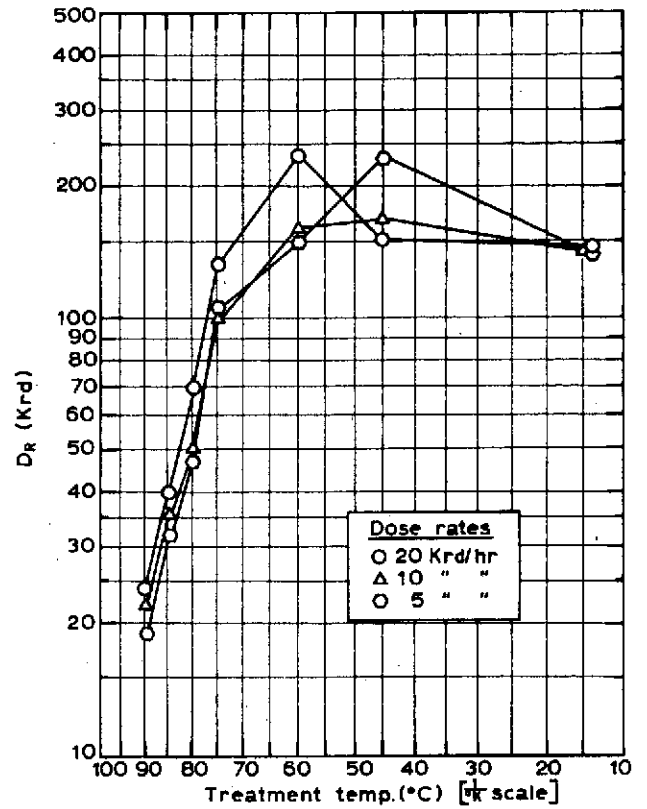
FIGURE 1.21: ARRHENIUS PLOT OF THE D-VALUE (TOTAL DOSE) FOR RADIATION INDUCED DEATH FOR WET HEAT THERMORADIATION.
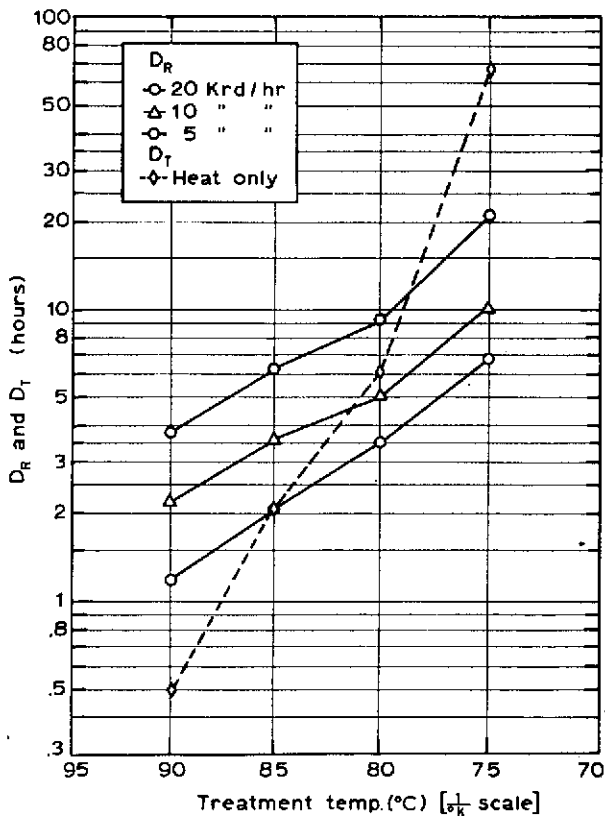
FIGURE 1.22: ARRHENIUS PLOT OF THE D-VALUE FOR RADIATION INDUCED DEATH AND THE D-VALUE FOR THERMALLY INDUCED DEATH FOUND IN WET HEAT THERMORADIATION FOR $75° \leq T \leq 90°C$.

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Table I.2

Energy of activation for the radiation induced D-value and the thermal induced D-value $75° \le T \le 90°C$

Reaction constant	Radiation level	$E_a \quad \dfrac{Kcal}{mole}$
D_R	20 Krd/hr	28.9
D_R	10 Krd/hr	24.85
D_R	5 Krd/hr	28.2
D_T	0 Krd/hr	65.3

Conclusions

For the completed wet heat thermoradiation study, different radiation induced mechanisms are in effect over the low and over the higher temperature ranges. The mechanism over the higher range combines properties of the low temperature radiation and thermal death characteristics and resembles the synergistic effect reported by Sandia Laboratories.

Future Work

We are proceeding to determine survivor curves for several dry heat thermoradiation conditions. Analysis work on possible origins of the synergistic effect continues.

PROJECT PERSONNEL

The following personnel all made major contributions to this project: Rebecca Gove, Donald Fisher, Irving Pflug, and Stephen Znameroski.

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2. DRY HEAT DESTRUCTION OF BACTERIAL SPORES

A. STUDY OF DESTRUCTION RATES AT LOW LEVELS OF WATER AT 90, 110, and 125°C.

Ronald L. Jacobson and Irving J. Pflug

INTRODUCTION

The destruction rates of Bacillus subtilis var. niger spores on surfaces heated in a clean room at 22°C, 35% RH and in a dry environment were summarized in Progress Report #5, page 29, These studies are an extension of the previous studies using an environmental system where it was possible to closely control the atmospheric water content in the test apparatus over a range of conditions from about 5 to 13,000 ppm (at 110°C the relative humidity would range from .0007% to .7%).

OBJECTIVES

The objectives of this series of experiments were (1) to obtain estimates of dry heat destruction rates (D-values) for Bacillus subtilis var. niger spores on surfaces in an open system at controlled low levels of water in the atmosphere surrounding the spores during heating at temperatures of 90, 110 and 125°C, and (2) to gather information that will allow us to model dry heat destruction in the range of about 5 to 13,000 ppm. Important questions we wanted to answer were: What is the relationship between the D-value and relative humidity calculated at test temperature? and What is the effect of test temperature on D-value at different water levels?

MATERIALS AND METHODS

Tests at about 10, 100, and 1000 ppm were carried out in a large glove box fitted with auxiliary systems to control and monitor the environment in the glove box. In addition, tests were carried out in the clean room at about 13,000 ppm. The environmental control system is described in Appendix A of this report.

A moisture monitor is an integral part of the moisture control system in the glove box. The unit of measure for water content is parts per million (ppm) by volume of water. Relative humidities for the several test conditions are given in Table 2A.1.

Table 2A.1

Relative Humidity as a Function of Hotplate Temperature and Parts Per Million Water in the Atmosphere

Hotplate Temperature °C	Relative Humidity (%)			
	10 ppm a)	100 ppm	1000 ppm	13,000 ppm b)
90	0.00145	0.0145	0.145	1.88
110	0.000707	0.00707	0.0707	0.919
125	0.000437	0.00437	0.0437	0.567

a) Calculated at standard atmospheric pressure--760 mm of Hg

b) Tests in clean room at 22°C, 50% RH

Bacillus subtilis var. niger spores (AAGF) suspended in deionized distilled water were deposited on stainless steel planchets. The aliquot size was 0.02 ml with approximately 10^6 viable spores. Deposition was made in the clean room (22°C, 50% RH). Shortly after deposition the inoculated planchets on boats set on trays were transferred to the glove box. They were conditioned in the glove box antechamber for about 20 minutes and then transferred to the main chamber. The spores remained in the main chamber of the glove box (29°C) for a conditioning period of about 18-20 hours. The moisture content of the glove box was monitored and controlled.

Heating tests were carried out using the planchet-boat-hotplate system described in Progress Report #3. Two tests were conducted simultaneously on two hotplates in the glove box.

The standard procedure was to use two planchets per boat (two

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experiments, SM2201 and SM2208, were conducted with only one planchet per
boat.) Both planchets from the same boat were placed in the same flask
and processed together. Each test consisted of four different heating
times with four boats per heating time. The surface of the hotplate was
divided into four sections. At each heating time there was one boat in
each hotplate section.

Following heating the planchets were processed according to the
NASA procedures (NASA Standard Procedures for the Microbial Estimation
of Space Hardware, NHB 5340.1A, October 1968, page 3 and 31) as outlined
in Progress Report #3, page 60.

RESULTS AND DISCUSSION

The data were plotted as semi-logarithmic survivor curves and the
D- and IR-values determined. Survivor curves are shown in Figures 2A.1
through 2A.6. The data are summarized in Table 2A.2.

The survivor curves obtained appear to be linear when y the log
number of survivors was plotted as a function of x the heating time.

At all three test temperatures (t) it appears that as the relative
humidity (RH) of the gas surrounding the spores decreases, the D_t-value
decreases. The D_t-values decrease by one log; for example, from 100 to
10 minutes or from 10 to 1 minute as the RH decreases from about 1.0%
to 0.0001%.

All the data do not fall on three perfectly parallel lines where
intercept values produce identical z-values; however, the data do align
themselves in such a way that we believe the only conclusion that is
warranted is that the data do form parallel lines and that the z-value
is probably a constant value both over the range of temperature and
relative humidities studied.

Before we calculate the z-value we must resolve the question of
the correct procedure for calculating a dry heat z-value when
test relative humidity is a variable. Examination of the data suggest
that if the lines are parallel then the magnitude of the z-value calculated
will vary according to the method used to select comparable values.
Should we use D_t-values at the same RH to calculate z? Or is z more meaning-
ful if D_t-values are selected some other way? In this report we have
selected D_t-values for each temperature at the same RH. For the data in
this experiment the average z-value is about 20°C.

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Table 2A.2

Data Summary for Dry Heat Destruction Tests of <u>Bacillus</u> <u>subtilis</u> var. niger
Spores on Surfaces at Controlled Low Levels of Water in the Surrounding Atmosphere

Experiment number	Heating temp. °C	ppm water in gas surrounding the spores	RH at test temp. %	No. of heating times	No. of data points	D_T (min)	95% conf. limits of D_T (min)	Time range first to last data point (min)	IR
RJ3032A	89	5.5	.0008	4	16	256	232–284	250–1000	.76
RJ3038C	90	5.0	.0007	4	16	255	236–278	240–960	.81
SM2214A	89	77	.01	2	8	434	386–497	720–1440	.95
SM2222B	90	75	.01	4	16	647	549–788	600–2400	.92
SM2227A	89	870	.1	4	16	1690	1566–1836	600–2400	.99
RJ3052A	90	13000	1.9	3	12	1741	1598–1911	2880–8640	1.05
RJ3073A	90	13000	1.9	4	16	2119	1978–2282	2160–8640	1.04
RJ3032B	108	5.5	.0004	4	16	20.6	18.1–23.9	20–80	.86
RJ3038B	109	5.0	.0004	4	16	22.2	20.7–24.0	20–80	.87
RJ2350A	109	20	.001	4	16	24.0	22.2–26.1	20–80	.86
SM2201A	109	88	.006	2	7	35.3	29.9–43.0	60–120	.95
SM2208B	108	103	.008	3	12	38.5	34.5–43.5	60–180	1.03
SM2214B	108	77	.006	2	8	32.8	28.3–38.8	60–120	1.01
SM2226B	108	870	.07	4	16	77.7	66.7–93.1	70–240	1.03
SM2227B	108	870	.07	4	16	87.6	75.4–104	60–240	1.00
RJ3052B	109	13000	.9	3	12	151.0	127–185	180–540	.85
RJ3073B	110	13000	.9	4	16	265.0	181–496	150–600	.97
RJ3032C	122	5.5	.0004	4	16	4.45	3.94–5.11	4–6	.87
RJ3038A	123	5.0	.0002	4	16	4.35	3.93–4.86	4–16	.91
RJ2350B	123	20	.0009	4	16	5.23	4.74–5.84	5–20	.88
SM2201B	123	88	.004	2	7	6.10	3.11–172	10–20	.90
SM2208A	123	103	.004	3	12	6.24	5.44–7.31	10–30	1.00
SM2222A	123	95	.004	4	16	8.13	7.09–9.51	10–40	.99
SM2226A	123	870	.04	4	16	13.3	12.5–14.3	10–40	1.02
RJ3052C	124	13000	.6	2	8	14.3	11.0–20.6	30–60	.90
RJ3073C	124	13000	.6	4	16	18.2	16.4–20.6	15–60	.95

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42441FEB90C LHP AAGF 7PPMRJ3032A

4144 8-9-72 90C 100PPM   SM2222B

42442-7-73 90C LHPGBAAGF RJ3038C

1224 8-1-72   90C 100PPM   SM2214A
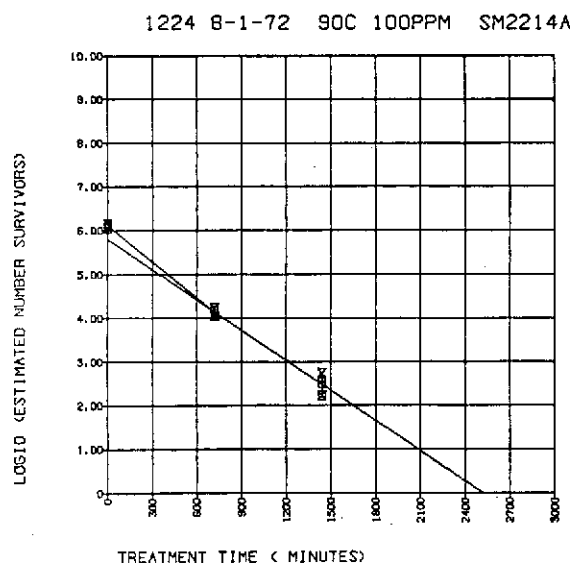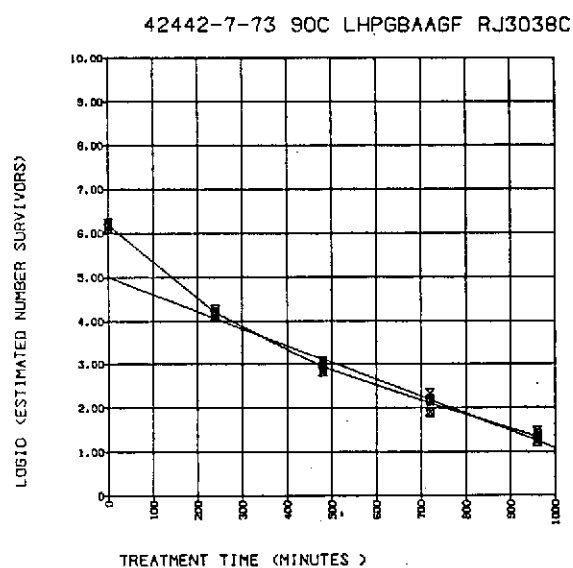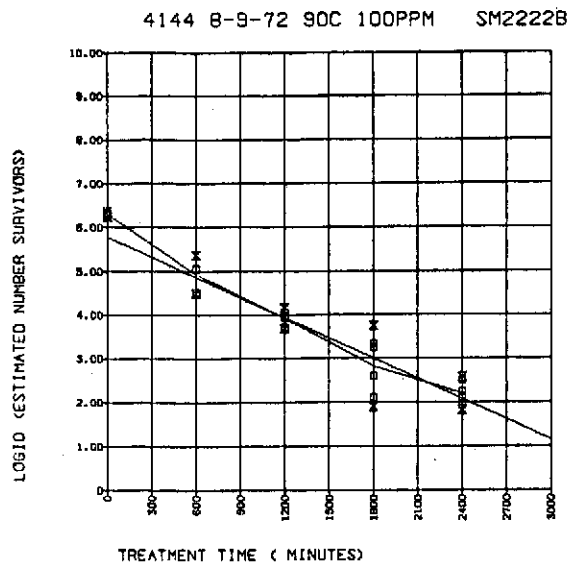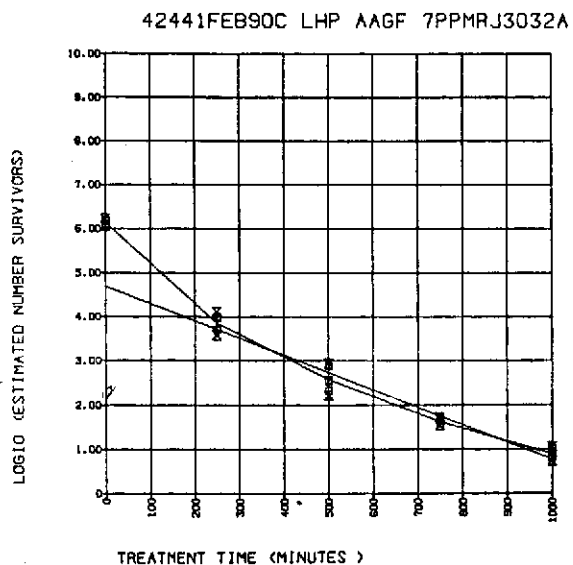
FIGURE 2A.1: SURVIVOR CURVES FOR TEST NUMBERS RJ3032A, RJ3038C, SM2214A, SM2222B AT 89°C AND 90°C.

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2244 8-16-72 90C 1000PPM SM2227A

LOG10 (ESTIMATED NUMBER SURVIVORS)

TREATMENT TIME (MINUTES)
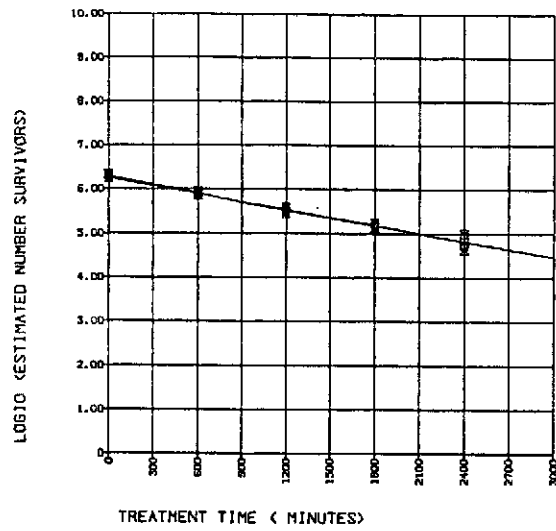
44342-21-73 90C CRHP AAGFRJ3052A

LOG10 (ESTIMATED NUMBER SURVIVORS)

TREATMENT TIME (MINUTES)
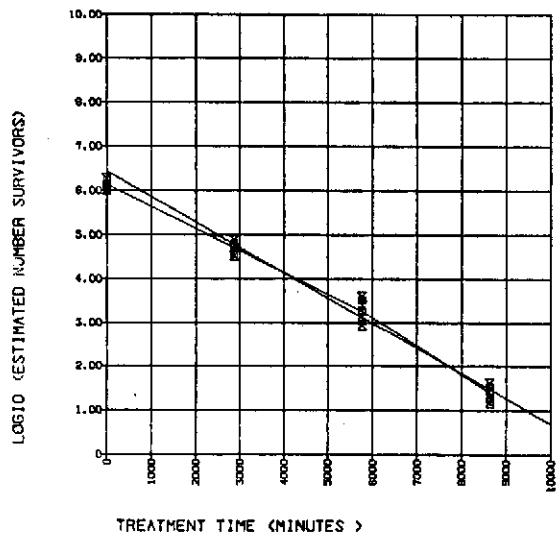
44443-14-73 90C CRHPAAGF RJ3073A

LOG10 (ESTIMATED NUMBER SURVIVORS)

TREATMENT TIME (MINUTES)
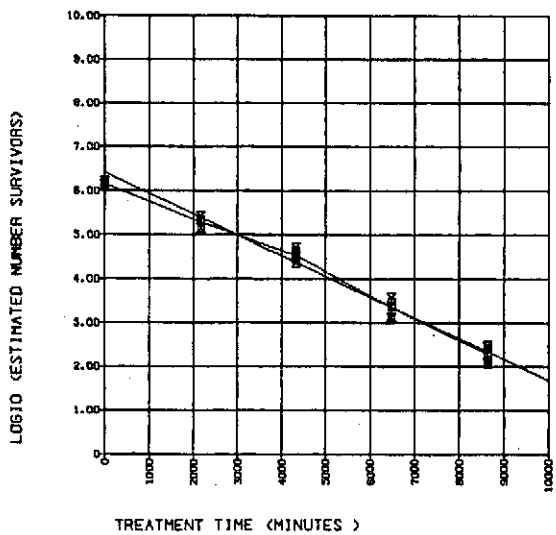
FIGURE 2A.2: SURVIVOR CURVES FOR TEST NUMBERS SM2227A, RJ3052A, RJ3073A AT 89°C AND 90°C.

24
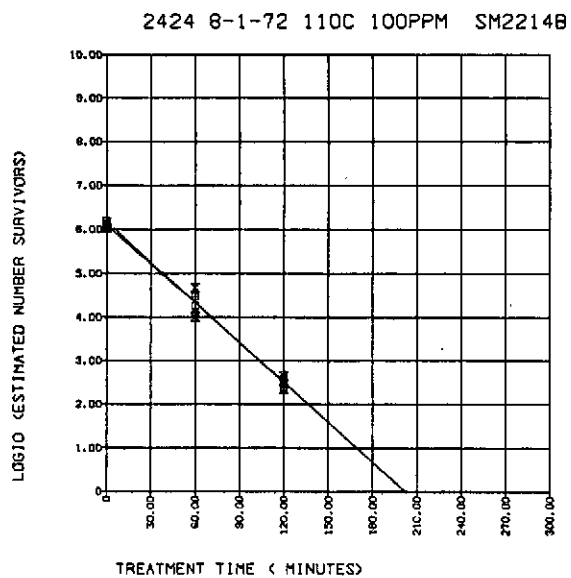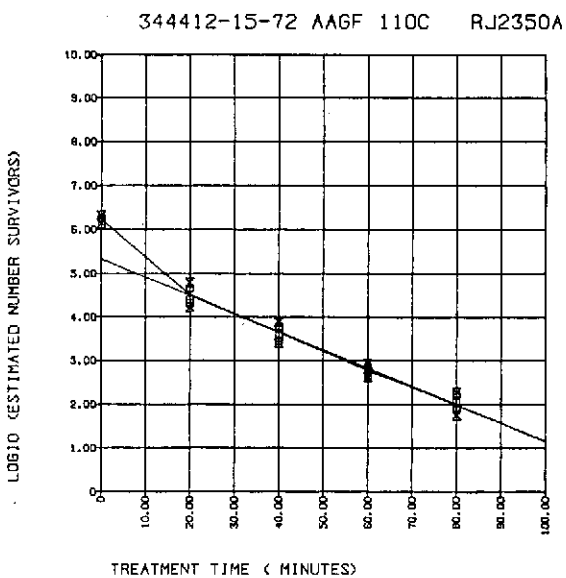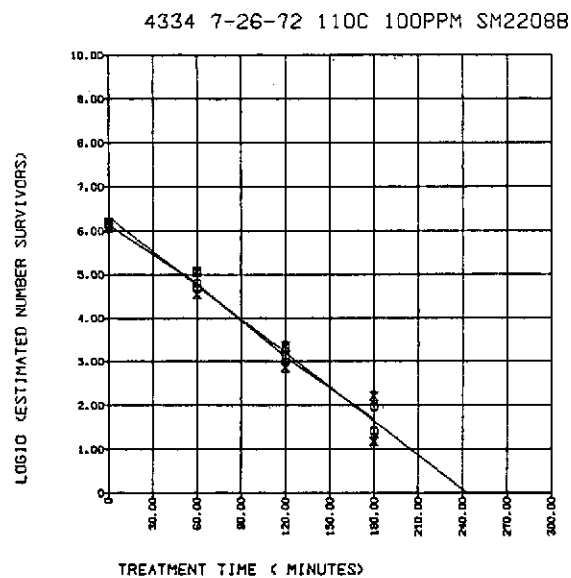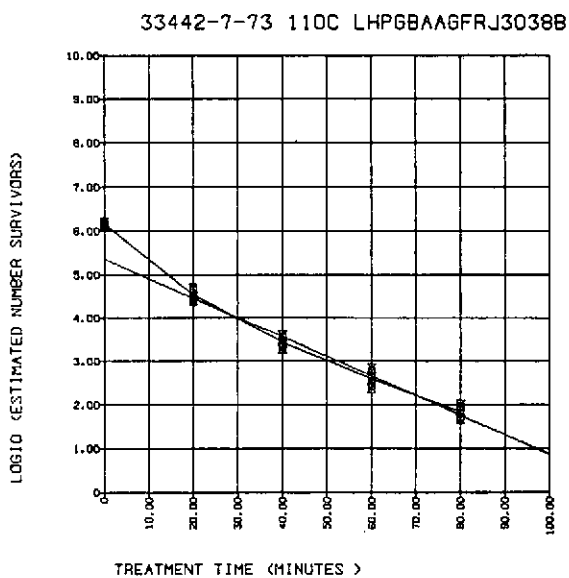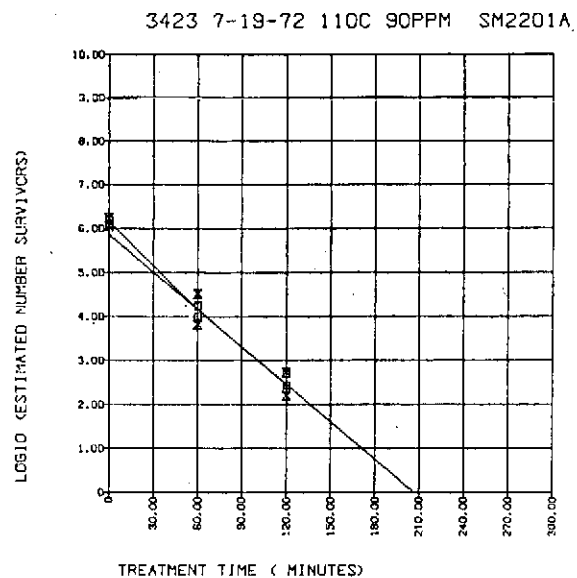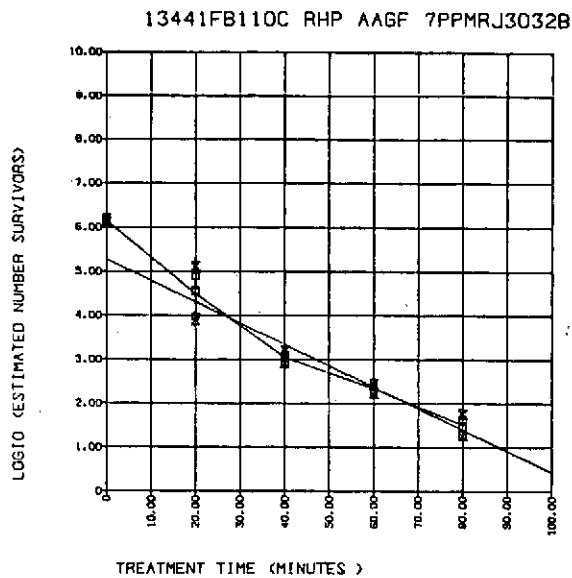
FIGURE 2A.3: SURVIVOR CURVES FOR TEST NUMBERS RJ3032B, RJ3038B, RJ2350A, SM2201A, SM2208B, SM2214B AT 108°C AND 109°C.

4442 8-15-72 110C 1000P SM2226B

LOG10 (ESTIMATED NUMBER SURVIVORS)

TREATMENT TIME (MINUTES)

14443-14-73 110C CRHPAAGFRJ3073B

LOG10 (ESTIMATED NUMBER SURVIVORS)

TREATMENT TIME (MINUTES)

23448-16-72 110C 1000PPM SM2227B

LOG10 (ESTIMATED NUMBER SURVIVORS)

TREATMENT TIME (MINUTES)

14342-21-73 110C CRHPAAGFRJ3052B

LOG10 (ESTIMATED NUMBER SURVIVORS)

TREATMENT TIME (MINUTES)

FIGURE 2A.4: SURVIVOR CURVES FOR TEST NUMBERS SM2226B, SM2227B, RJ3052B, RJ3073B AT 108°C AND 109°.

26
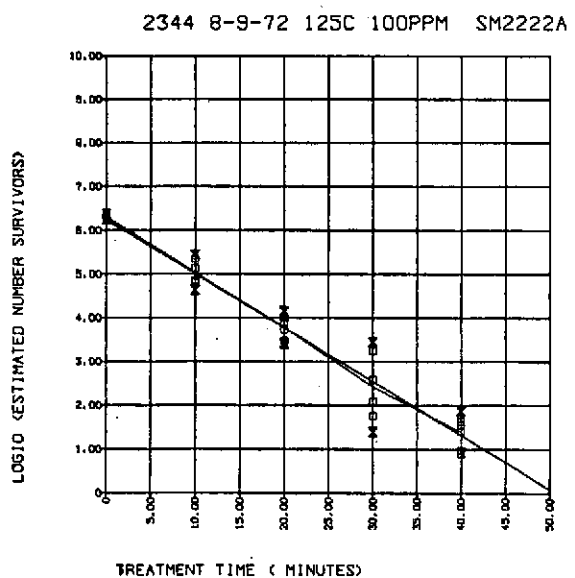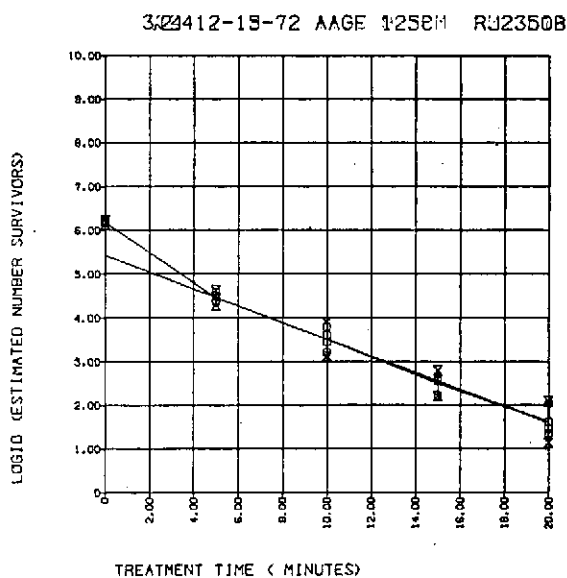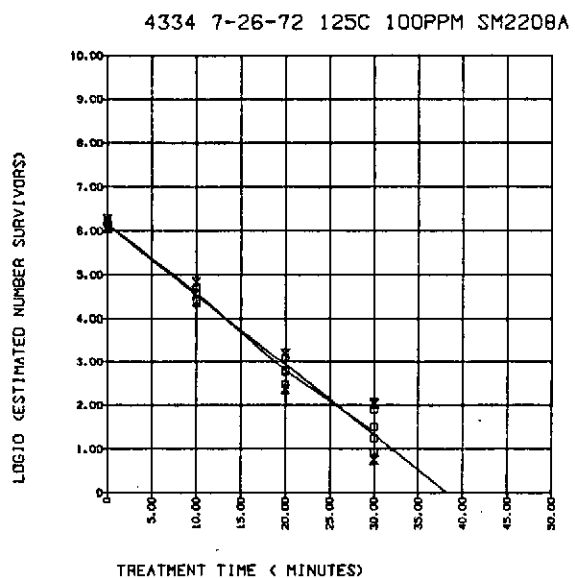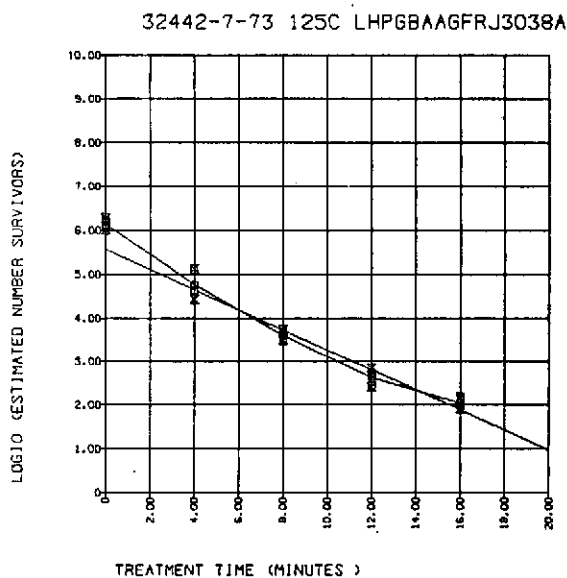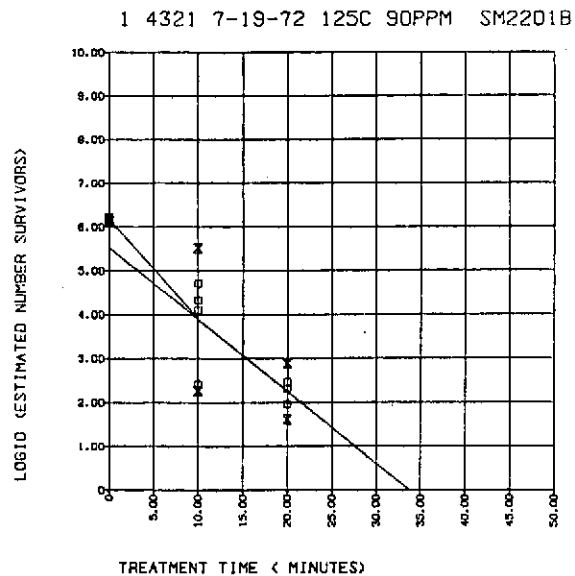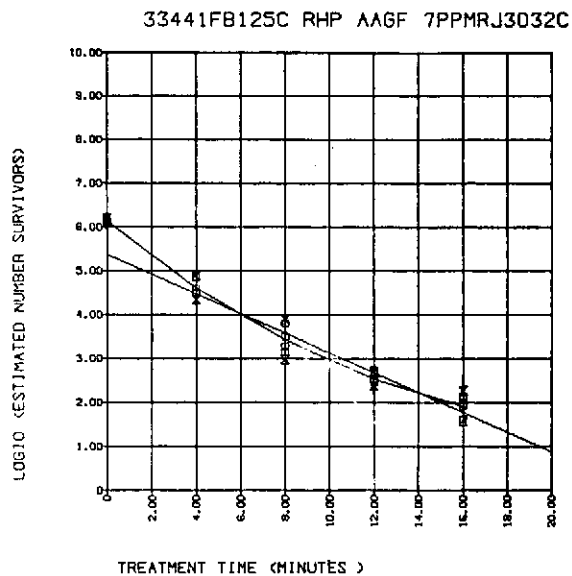
FIGURE 2A.5: SURVIVOR CURVES FOR TEST NUMBERS RJ3032C, RJ3038A, RJ2350B, SM2201B, SM2208A, SM2222A AT 122°C AND 123°C.

4442 8-15-72 125C 1000P SM2226A

LOG10 (ESTIMATED NUMBER SURVIVORS)

TREATMENT TIME (MINUTES)
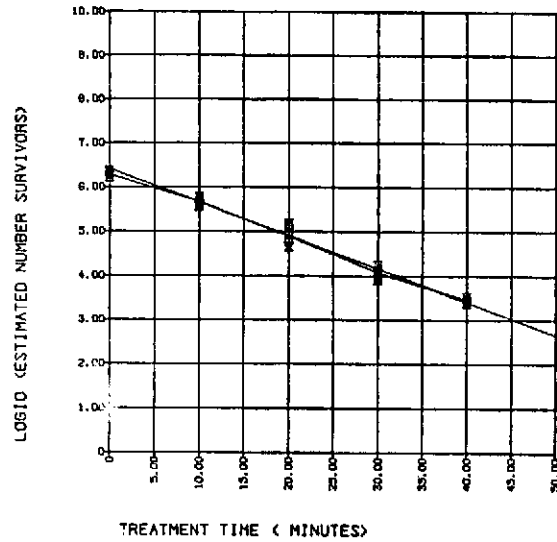
11242-21-73 125C CRHPAAGFRJ3052C

LOG10 (ESTIMATED NUMBER SURVIVORS)

TREATMENT TIME (MINUTES)
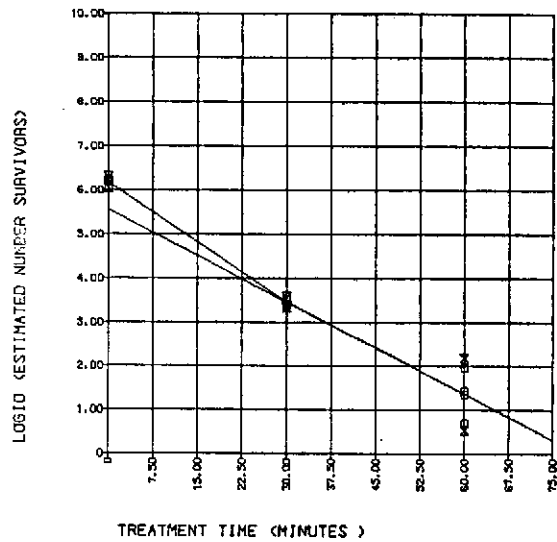
12443-14-73 125C CRHPAAGFRJ3073C

LOG10 (ESTIMATED NUMBER SURVIVORS)

TREATMENT TIME (MINUTES)
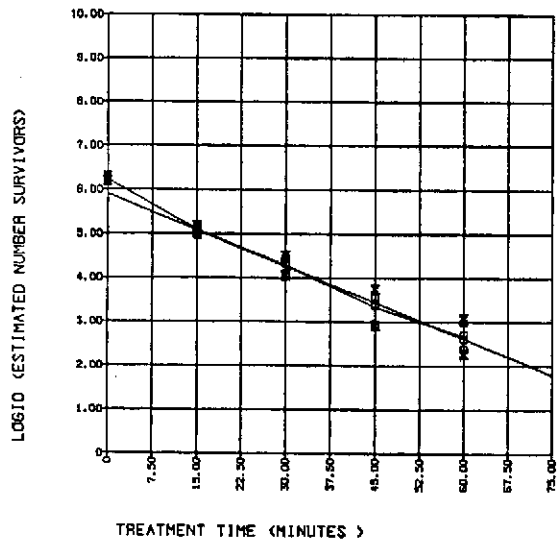
FIGURE 2A.6: SURVIVOR CURVES FOR TEST NUMBERS SM2226A, RJ3052C, RJ3073C AT 123°C AND 124°C.

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Examination of the intercept ratios (IR) reveals some interesting trends. In Table 2A.3 are shown IR values for the four water levels and three test temperatures. There appears to be a trend for the IR to decrease as ppm water decrease from 1000 to 100 to 22 or 5 ppm. Since spores were equilibrated at these moisture levels at about 29°C what we are observing is a decrease in IR with a decrease in spore water. This phenomenon has been previously observed by Drummond and Pflug and by others. There appears to be a weak trend for IR to increase as test RH increases. The IR values for the highest water conditions 13,000 ppm are higher at 90°C. These tests were carried out in the clean room rather than in the environmental chamber which may be the cause of the differences.

Table 2A.3

Intercept Ratios for the Several Test Temperatures and Water Levels

Water Level	Intercept Ratios[a] at Test Temperatures		
	89°C	109°C	123°C
less than	.76	.86	.87
25 ppm	.81	.87	.91
		.86	.88
100 ppm	.95	.95	.90
	.92	1.03	1.00
		1.01	.99
1000 ppm	.99	1.03	1.02
		1.00	
13,000 ppm	1.05	.85	.90
	1.04	.97	.95

[a] Intercept ratio (IR) = $\log y_0 / \log N_0$

CONCLUSIONS

Following are the conclusions regarding dry heat destruction of Bacillus subtilis var. niger spores below 2% RH at test temperature:

1. D-values decrease continuously as the relative humidity at test temperature decreases.

2. When the log of the D-value is plotted as a function of the log of RH the data at each test temperature appear to form straight lines, i.e., the lines appear to be parallel

3. A decrease in RH from 1.0% to 0.0001% reduces the D_t-value by 90%.

4. The z-value is about 20°C and appears to be a constant over the temperature and RH range studied.

PROJECT PERSONNEL

The following personnel all made major contributions to this project: Rebecca Gove, Ronald Jacobson, Susan Maki, Yvonne Thun, and Irving J. Pflug.

2. DRY HEAT DESTRUCTION OF BACTERIAL SPORES

B. ENCLOSED IN GLASS JARS

Geraldine M. Smith, Ronald Jacobson, and Irving J. Pflug

INTRODUCTION

The Spacecraft Bioassay Laboratory, Kennedy Space Center with support from the PHS, CDC-Phoenix group, and the FDA-Cincinnati group are involved in carrying out a large project to develop a better understanding of the dry heat destruction characteristics of organisms that appear on fallout-strips placed in spacecraft assembly areas. The basic vehicle for collection of the microbial fallout was Teflon strips 3 inches wide and 6 feet long that after exposure were rolled up and placed in glass Mason jars. The Teflon strip in the Mason jar was in turn placed in a dry heat oven and subjected to a sterilization cycle. Variation in the results of some of the early studies led us after consultation with the Planetary Quarantine Officer to carry out the experiments described below.

MATERIALS AND METHODS

Bacillus subtilis var. niger spores (AAGF) were deposited on stainless steel planchets and allowed to equilibrate for 18 hours in the clean room (22°C, 50%RH). Each deposit contained about 10^6 spores. The test planchets were placed in one pint Mason jars and heated in a circulating air oven. The control planchets were heated using the planchet-boat-hotplate system described in Progress Report #3. The oven and the hotplate were operated at 125°C and were located in the clean room.

Test SM2180

Four jars placed in a horizontal position (Figure 2B.1) each containing two inoculated 1" X 2" stainless steel planchets were heated at each time interval. One planchet was located near the lid of the jar and one near the bottom. The lids on two of the four jars were closed tightly; the lids on the other two jars were closed without tightening (loosely closed).

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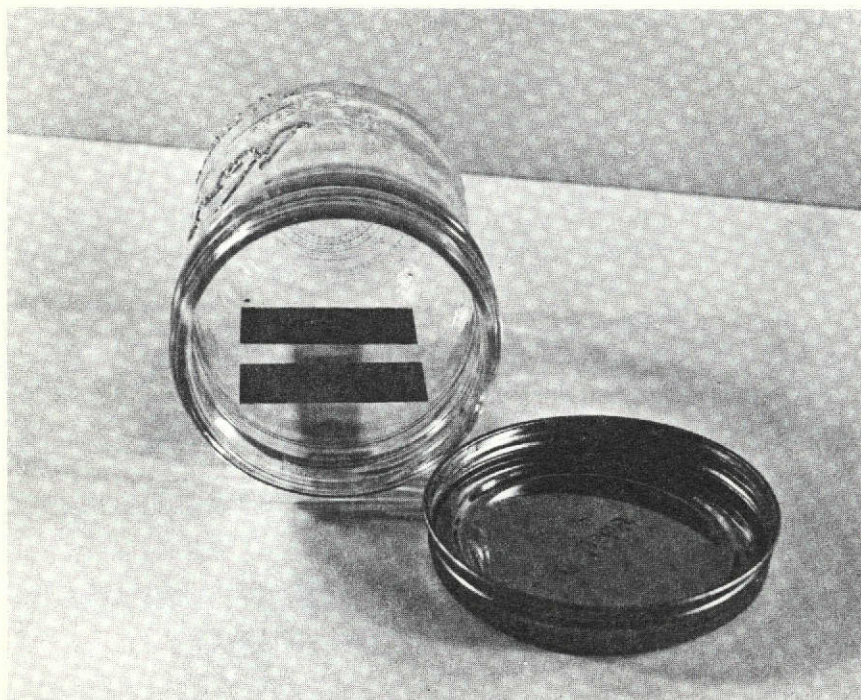
Figure 2B.1: Planchets (1" X 2") in Mason Jar

Test SM2202

Three jars in a horizontal position each containing two inoculated
1" X 2" planchets were heated at each time interval. The lid was applied
in reverse; the rubber gasket around the edge of the Kerr lid was facing
away from the jar. The jars were all loosely closed. The jars were
placed in the oven with the lids facing away from the oven door.

Tests SM2209, 2217, and 2252

Teflon strips 24" X 3 1/8" X .01" were coiled into a 2 1/2"
diameter roll. The roll of Teflon was held in place by three screws.
Each roll consisted of three layers of Teflon. Stainless steel planchets
1/2" X 1/2" were curved to fit the arc of the Teflon roll. Teflon
covers 3/4" X 3/4" were made to fit over the curved planchets.

The Mason jars, Kerr lids, the Teflon rolls and Teflon covers
were sterilized at 175°C in an oven for three hours and then immediately
placed in the clean room to equilibrate for 24 hours. Sterile forceps
were inserted between the layers of the Teflon roll in order to expose
all layers to the clean room atmosphere. Immediately before the heat
treatment, the covers were placed over the inoculated planchets. Using
sterile forceps one covered, inoculated planchet was placed between
layers one and two of the roll and another covered, inoculated planchet
was placed between layers two and three of the roll. The roll was
inserted into the Mason jar and the jar was loosely closed, the rubber
gasket faced away from the jar. The jars were placed in the preheated
oven with the lids facing away from the oven door.

After heating, the jars were removed from the oven and both the
planchet and its cover were analyzed for survivors using NASA standard
procedures.

RESULTS

The results of Tests SM2180 and SM2202 are presented in Tables
2B.1 and 2B.2. In all cases, in these tests there were fewer survivors
on the planchets located near the lid of the jar than on the planchets
located near the bottom of the jar. The data for the individual planchets
heated in loosely closed and tightly closed jars are shown graphically
in Figures 2B.2 and 2B.3. The survivor curve for planchets located near
the bottom of loosely closed jars and the survivor curve for the control
planchets heated on the hot plate are shown in Figure 2B.4.

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Table 2B.1

Number of Survivors Per Planchet, Test SM2180

Heating time (min)	Tight lid		Loose lid	
	Near lid	Near bottom	Near lid	Near bottom
0	1,650,000	1,650,000	1,650,000	1,650,000
60	68,800 146,000	238,000 781,000	2,750 77,300	89,300 238,000
120	75 0	1,850 25	50 0	600 1,100
180	2.5 37.5	12.5 183	0 0	5 2.5

Table 2B.2

Number of Survivors Per Planchet, Test SM2202.
All Jars Had Loose Lids.

Heating time (min)	Near lid	Near bottom
0	1,310,300	1,310,300
30	195,075 341,700	395,250 749,700
60	21,250 1,025 0	44,250 1,275 0
90	0 0 20	5 33 1,050
120	0 5 0	2.5 51.2 12.5
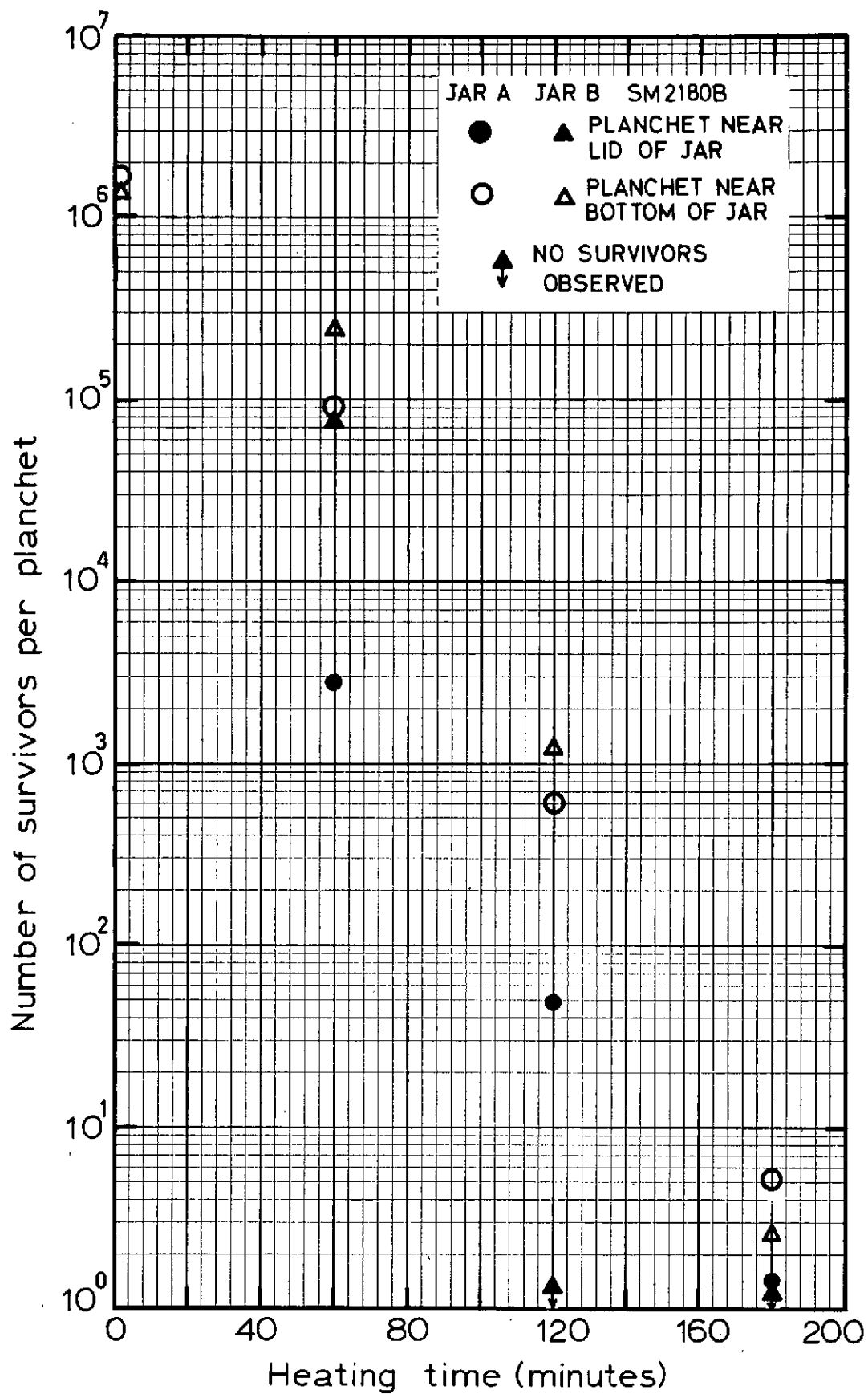
Figure 2B.2: Number of Surviving Bacillus subtilis var. niger Spores as a Function of Heating Time. Spores on Strips in Jars Heated at 125°C, Loose Lids.
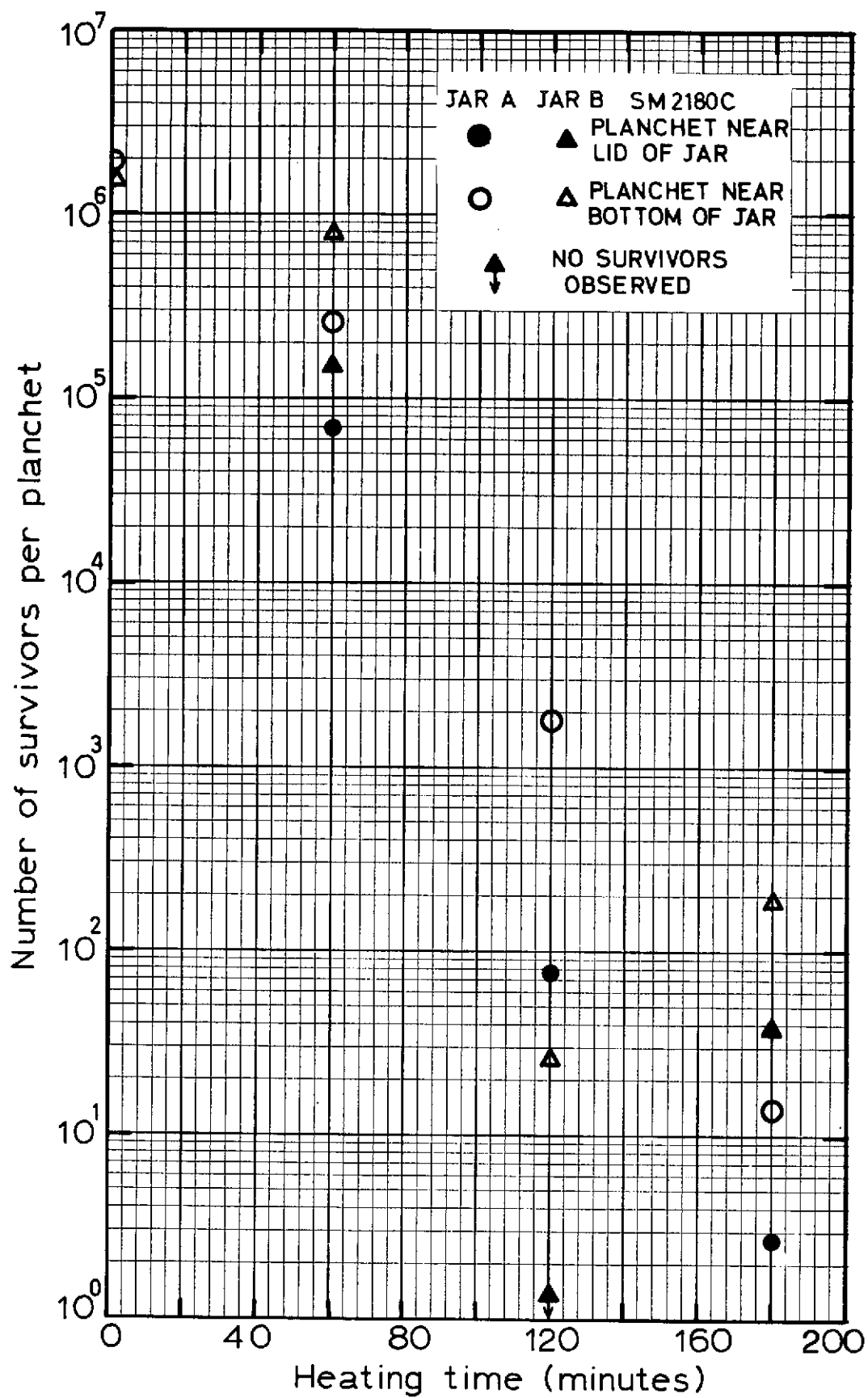
Figure 2B.3: Number of Surviving Bacillus subtilis var. niger Spores as a Function of Heating Time. Spores on Strips in Jars Heated at 125°C, Tight Lids.
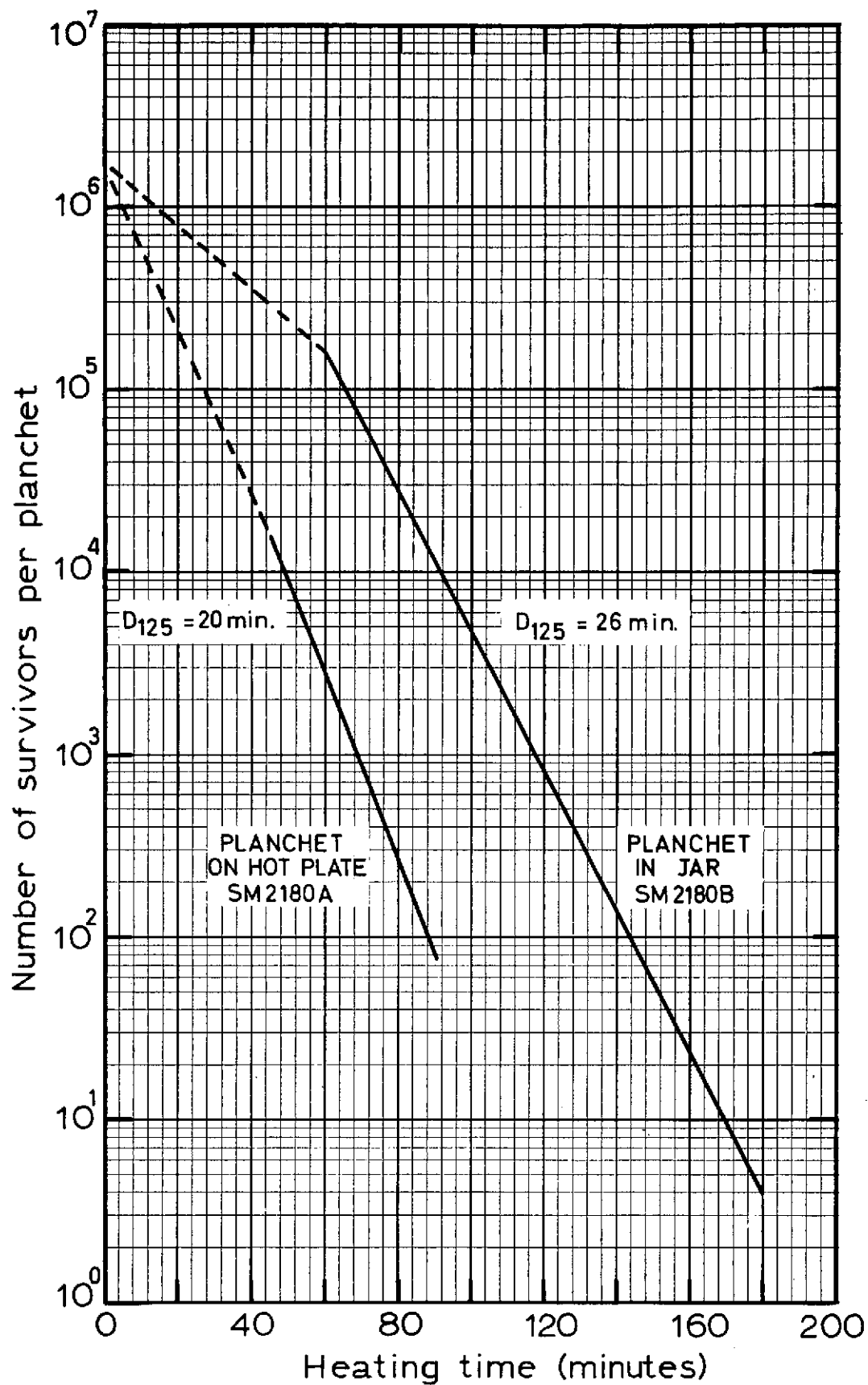
Figure 2B.4:   Survivor Curves for Bacillus subtilis var. niger Spores
on Planchets Near Bottom of Jar with Loose Lid and on Hot Plate.

The survivor curves for spores on planchets heated on the hot plate and corresponding survivor curves for spores on planchets between layers of a Teflon roll enclosed in a loosely closed jar are shown in Figures 2B.5, 2B.6, and 2B.7. The D-values and intercept ratios (IR) for these same tests are listed in Table 2B.3.

Table 2B.3

D-Values and Intercept Ratios of B. subtilis var. niger Spores on Planchets. Heated at 125°C on the Hot Plate and In Teflon Rolls Enclosed in Jars

Test No.	Planchet on Hot Plate		Planchet in Teflon Roll	
	D-value (min.)	Intercept Ratio (a)	D-value (min.)	Intercept Ratio (a)
SM2209	15	1.09	18	1.1
SM2217	18	1.02	22	1.12
SM2252	17	1.02	14	1.11

a $IR = \log Y_o / \log N_o$

DISCUSSION

The results of these tests using Bacillus subtilis var. niger spores indicate to us that there are widely varying conditions at different locations inside the same jar and also there are widely varying conditions among jars.

The greater spore survival near the bottom of the jar is very interesting but difficult to explain. Two unknowns are: (1) the movement of convection currents in the jar that may produce a difference in water vapor pressure in different parts of the jar, and (2) the effect of the metal lid. The relative tightness of the closure appears to have little effect. All jars were closed; therefore, since water vapor movement is by diffusion, this effect is not surprising.

In Tests SM2117 and SM2209, after 30 minutes there were almost one-log more spores surviving on planchets in jars than planchets on the hot plate. However, in Test SM2252B after 30 minutes, there was no meaningful differences in spore survival. Experience gained from other dry heat studies suggest that these differences were caused by differences in the rate of water loss either during conditioning or
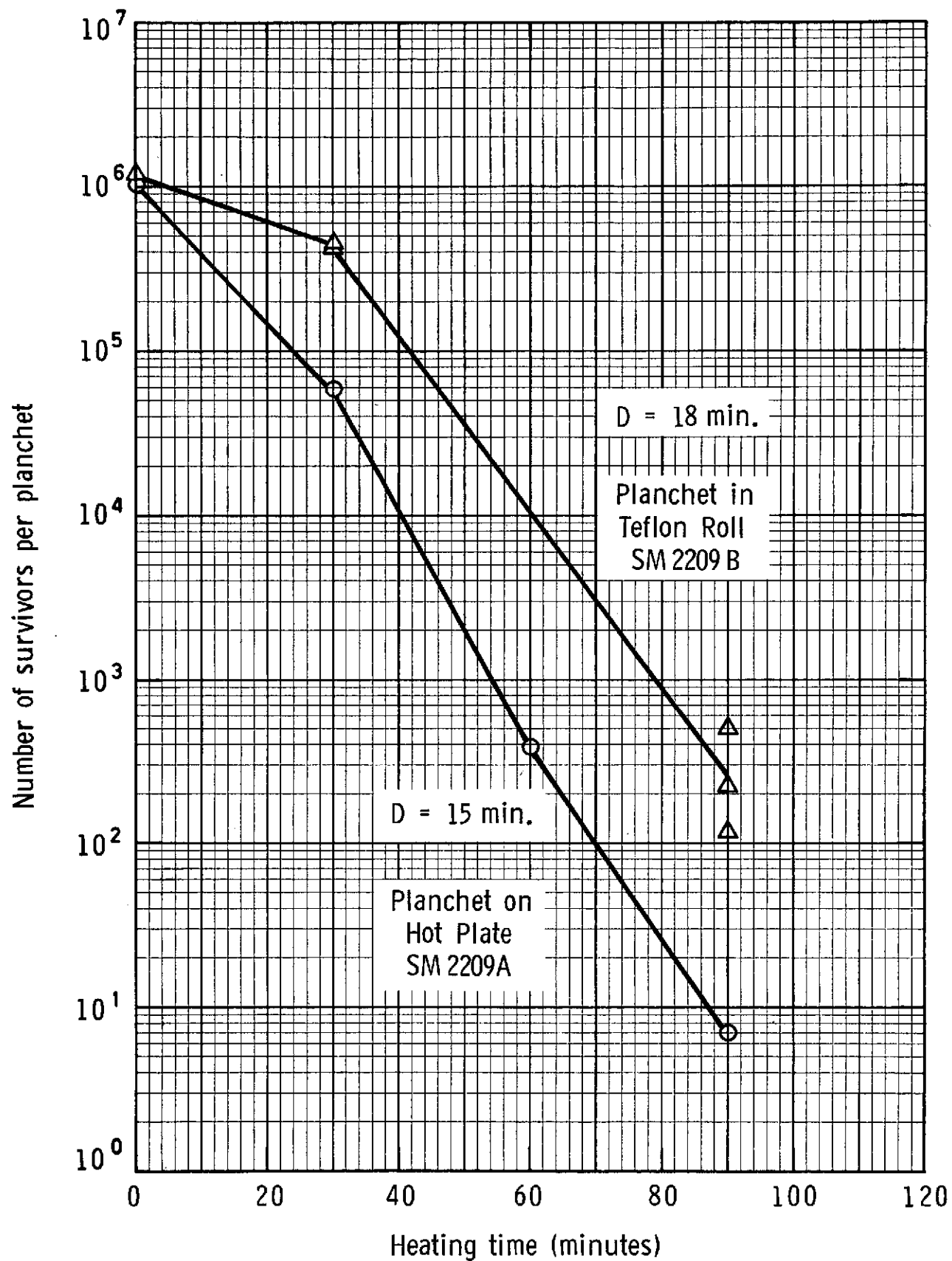
Figure 2B.5: Survivor Curves for Bacillus subtilis var. niger Spores on Planchets Heated at 125°C in Teflon Roll (SM2209B) Compared to Those Heated at 125°C on a Hot Plate (SM2209A).
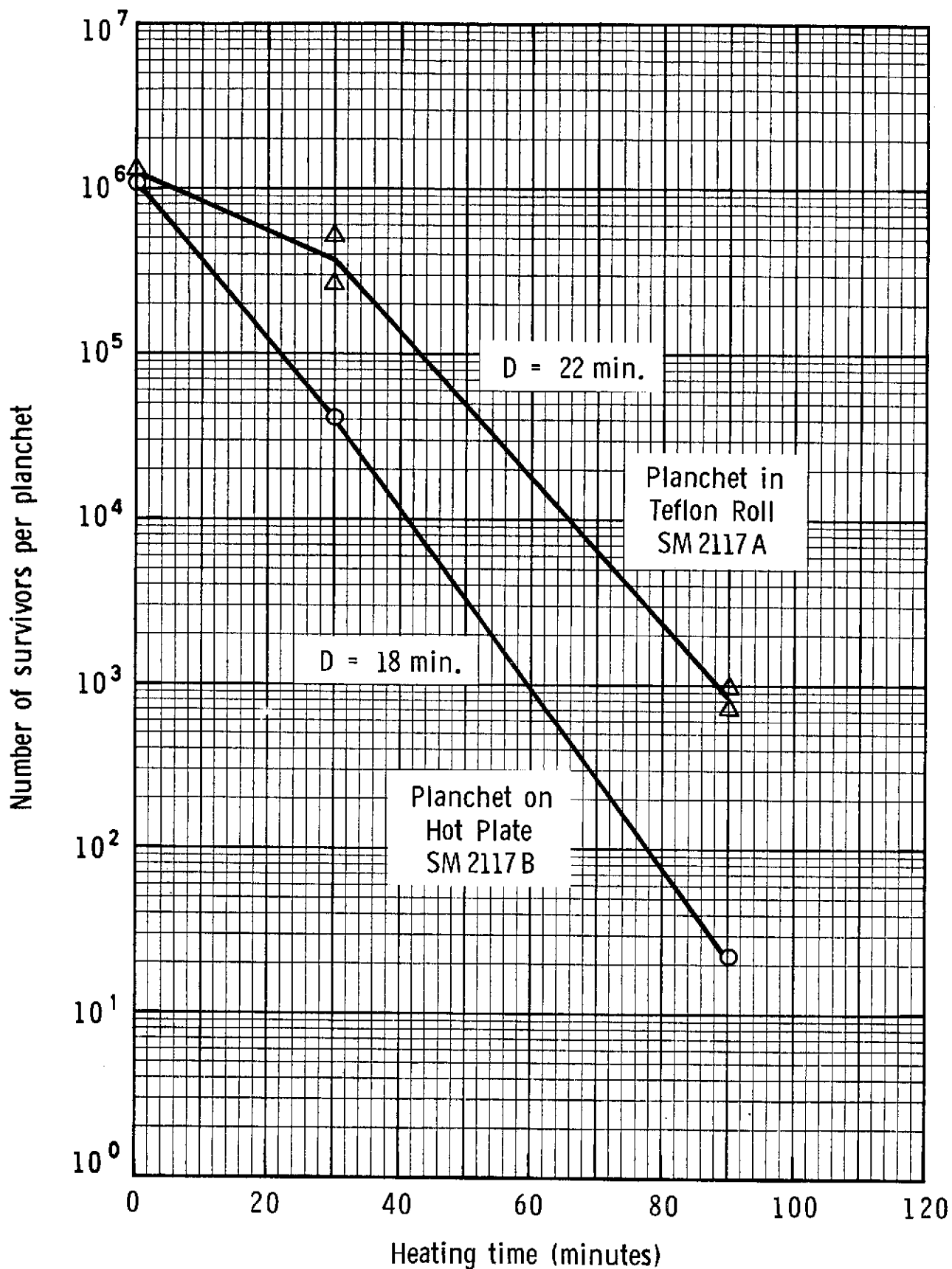
Figure 2B.6: Survivor Curves for Bacillus subtilis var. niger Spores
on Planchets Heated at 125°C In Teflon Roll (SM2117A)
Compared to Those Heated at 125°C on a Hot Plate (SM2117B).
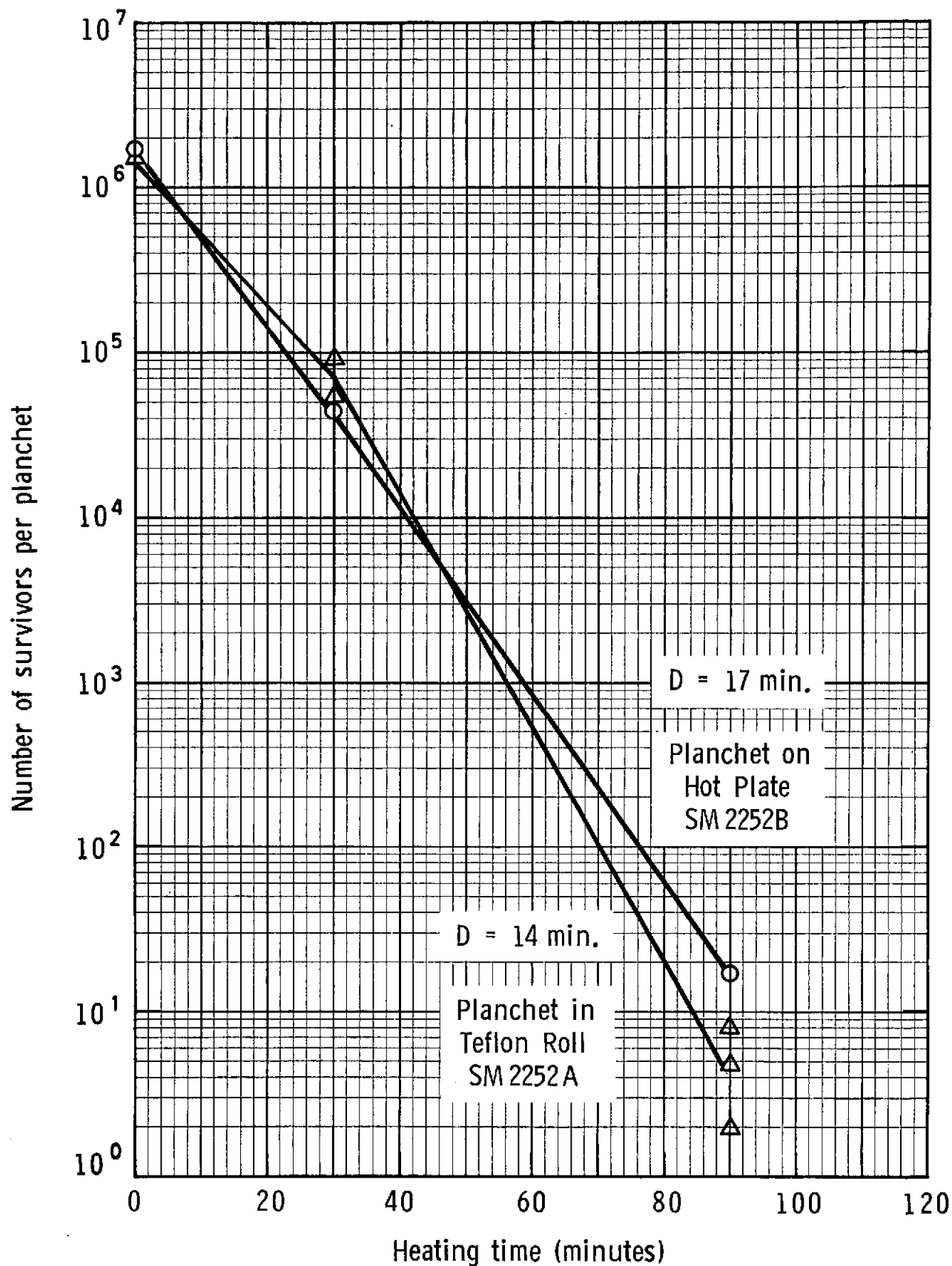
Figure 2B.7: Survivor Curves for Bacillus subtilis var. niger Spores on Planchets Heated at 125°C in Teflon Roll (SM2252B) Compared to Those Heated at 125°C on a Hot Plate (SM2252A).

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during the early part of the heating period.

The results indicate that the jar system has more variability as far as spore survival is concerned than the hotplate system. The range of D_{125}-values for spores on planchets in jars was eight minutes (from 14 to 22 min) while for spores on planchets heated on the hotplate, it was only three minutes (from 15 to 18 min).

This work was carried out to gather information regarding the effect of the jar system on spore survival and was supportive to work carried out by the Spacecraft Bioassay Laboratory, Kennedy Space Center.

The single important conclusion that can be drawn from these tests is that in a system where heating is carried out in jars, there is a large amount of variation in the lethality of the process as measured by Bacillus subtilis var. niger spores.

PROJECT PERSONNEL

The following personnel all made major contributions to this project: Sue Maki, Rebecca Gove, Ronald Jacobson, Geraldine Smith, Yvonne Thun, and Irving Pflug.

3. LABORATORY CONTROL AND STATISTICAL ANALYSIS

A. THE EFFECT OF DIFFERENT CLEANING METHODS AND STORAGE CONDITIONS ON THE DRY HEAT RESISTANCE OF BACILLUS SUBTILIS VAR. NIGER SPORES

Geraldine M. Smith and I.J. Pflug

INTRODUCTION

Each microbiological laboratory tends to develop and use its own preferred methods for growing, cleaning and storing spores. Since each laboratory is working on its own special problem, this is perhaps to be expected. This desire of each laboratory to use its own methods is so strong that recently an eminent microbiologist said facetiously "One microbiologist would rather use another microbiologist's toothbrush than to use his laboratory methods." It is generally accepted that culture methods have an effect on the dry heat resistance of bacterial spores (Lechowich and Ordal[1] and Williams and Harper[2]). However, there is very little data in the literature regarding the effect of cleaning methods and storage conditions on the long-term survival of these spores and their heat resistance after long periods of storage. To provide an answer to these questions a study has been initiated to determine the effect of spore crop cleaning procedure, suspending medium and storage temperature on the long-term survival and dry heat resistance of Bacillus subtilis var. niger spores.

OBJECTIVE

The objective of these experiments is to determine the optimum methods for the long-term storage of spores of Bacillus subtilis var. niger so there is maximum stability and minimum change. This is important in our overall testing program because all tests take place over a period of time and if we can minimize spore change with time we will have improved control of our experiments.

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[1] Lechowich, R.V. and Ordal, Z. John (1962): The Influence of the Sporulation Temperature on the Heat Resistance and Chemical Composition of Bacterial Spores. Can. J. of Microb., vol. 8, pp. 287-295.

[2] Williams, O.R. and Harper, O.F., Jr. (1951): Studies on Heat Resistance IV. Sporulation of Bacillus cereus in Synthetic Media and the Heat Resistance of the Spores Produced. J. Bact. vol. 61, pp. 551-556.

MATERIALS AND METHODS

A large spore crop was produced using SSM-10 broth; the procedure used in producing the spore crop was given on page 80 of Progress Report #3.

Two spore cleaning procedures were used: (1) <u>Dirty</u>: Spores were not cleaned. The spore crop produced in SSM-10 broth was centrifuged and decanted; it was resuspended in distilled water for water storage tests and resuspended in 95% ethanol for ethanol storage tests. (2) <u>Clean</u>: The spore crop produced in SSM-10 broth was subjected to an insonation treatment to free the spores and break up the vegetative cell debris followed by several washings with deionized distilled water. The spore preparation steps are diagrammed in Figure 3A.1.
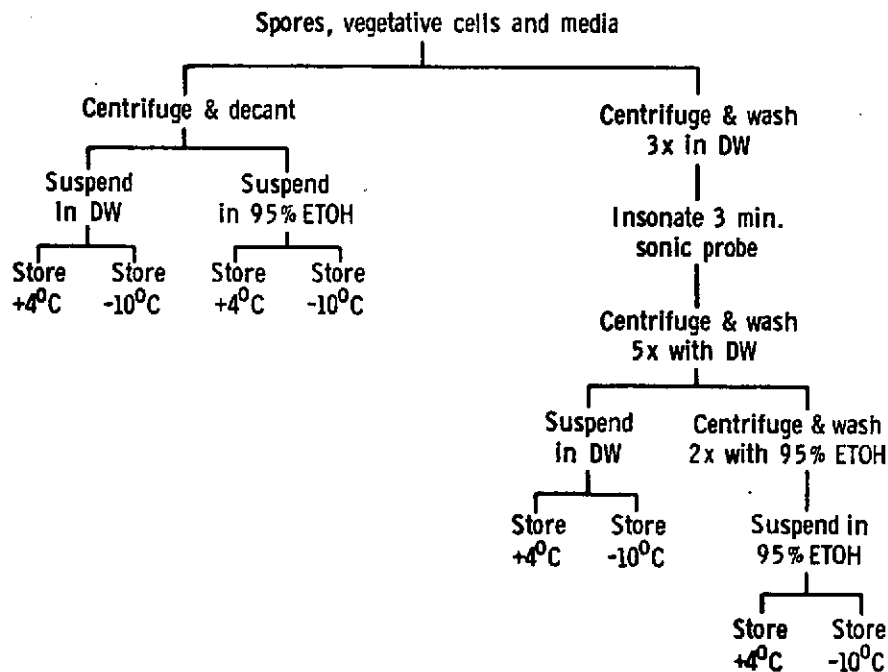
Figure 3A.1: Procedure Used to Prepare Spores of <u>Bacillus subtilis</u> var. <u>niger</u> for the Spore Storage Tests

The two suspending media, deionized distilled water and 95% ethanol were chosen because these are the two suspending media that seem to be in general use by microbiologists working in different laboratories. Good results have been reported both when using distilled water and when using 95% ethanol.

Two storage temperatures are being evaluated; 4°C, a standard storage temperature used generally in microbiological laboratories and frozen storage at -10°C. Again good results have been reported for both of these storage conditions.

To determine the dry heat survival characteristics of the Bacillus subtilis var. niger spores, it was arbitrarily decided to use test temperatures of 110° and 125°C at six-month intervals over a three-year period.

The eight storage conditions that are being evaluated are shown in Table 3A.1. Spores from each of the 8 storage conditions will be tested at 110 and 125°C.

Table 3A.1

Experimental Design

Stored at 4°C	Stored at -10°C
Dirty in water	Dirty in water
Dirty in 95% ethanol	Dirty in 95% ethanol
Clean in water	Clean in water
Clean in 95% ethanol.	Clean in 95% ethanol

The planchet-boat-hotplate method (described in Progress Report #3) of evaluating the heat resistance has been used and, since there were a large number of samples, only two heating times were selected for testing the performance of the spores after storage.

Planchets are inoculated with 10^6 spores and equilibrated for 18 hours in the clean room at 22°C, 50% RH before heating tests are initiated. Two boats each containing two planchets are heated at the short time period and three boats each with two planchets are heated for the long time period. Heating times were selected to give a one log decrease in numbers at the short heating time period and a four log reduction in the numbers of survivors at the long heating time period.

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RESULTS AND DISCUSSION

All the results for the tests carried out after three months storage are summarized in Tables 3A.2 and 3A.3.

Table 3A.2

D-values and Intercept Ratios for <u>Bacillus</u> <u>subtilis</u> var. <u>niger</u> Spores Stored Under Various Conditions for Three Months and Heated at 110°C

Test No.	Distilled Water				95% Ethanol			
	Clean		Dirty		Clean		Dirty	
	D(min)	IR[a]	D(min)	IR	D(min)	IR	D(min)	IR
	4°C Storage							
BG2278	229	.96	296	.92	354	1.00	593	1.01
	-10°C Storage							
BG2292	292	1.00	218	.91	247	1.12	262	1.20
BG2299	297	.99	272	.88	223	1.07	311	1.04

[a] $IR = \log Y_o / \log N_o$

Heating at 110°C

For spores stored at -10°C the resistance of the spores in water and in ethanol was of the same order of magnitude. Differences between clean and dirty spores at -10°C were small, although dirty spores in ethanol were more resistant than clean spores. Spores stored in ethanol at 4°C showed a much larger D-value than those stored in water at 4°C. For both water and ethanol suspended spores at 4°C the dirty spores had larger D-values than the clean spores.

Table 3A.3

D-values and Intercept Ratios for Bacillus subtilis var. niger Spores
Stored Under Various Conditions for Three Months and Heated at 125°C

| Test No. | Distilled Water | | | | 95% Ethanol | | | |
| | Clean | | Dirty | | Clean | | Dirty | |
	D(min)	IR[a]	D(min)	IR	D(min)	IR	D(min)	IR
	4°C Storage							
BG2278	30	.92	32	1.01	44	1.00	65	1.02
	-10°C Storage							
BG2292	39	.99	52	.86	40	1.06	57	1.00
BG2299	39	.99	48	.92	31	1.05	41	1.00

[a] $IR = \log Y_o / \log N_o$

Heating at 125°C

Comparing the D-values of clean versus dirty spores for both the distilled
water and the ethanol storage treatments, we find that in all conditions
the D-values for dirty spores were greater than for the comparable clean
spores. The increase of D-value for dirty over the D-value for clean ranged
from about 10% to 50%.

Spores stored in distilled water at -10°C were higher in resistance than
those stored at 4°C. The difference was greater for the dirty spores than
for the clean spores. It appears that the -10°C storage enhanced the resis-
tance of the dirty spores more than it did the clean spores.

Dirty spores stored in 95% ethanol had larger D-values than clean spores.
D-values were larger for 4°C than for -10°C storage. The increase of the
4°C storage over the -10°C storage was in the range of 10 to 20%.

Intercept Ratios (IR)

The IR values of the spores stored in ethanol were 1.0 or larger,
although they varied from a low of 1.0 to a high of 1.2. For spores stored
in water, the IR values were all less than 1.0 except for one test of clean
spores stored at -10°C and heated at 110°C where the IR value was 1.0 and
one test of dirty spores stored at 4°C and heated at 125°C where the IR
value was 1.08. We believe that this difference is meaningful but do not
know its significance at this time.

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FUTURE WORK

This study will continue and will be reported on in future progress reports.

PROJECT PERSONNEL

The following personnel all made major contributions to this project: Rebecca Gove, Ronald Jacobson, Sue Maki, Irving Pflug, Geraldine Smith, and Yvonne Thun.

3. LABORATORY CONTROL AND STATISTICAL ANALYSIS

B. MEDIA PREPARATION AND QUALITY CONTROL

Ronald Jacobson, Richard Holcomb, and Bliss Moore

Uniformity in growth inducement potential between batches of media is important when comparing results of different experiments. If one batch of media causes more or less growth than another for similarly treated spores, differences in observed results would not be due to treatment but due to recovery methods. The following test was conducted for the purpose of determining if there were differences between batches of media.

MATERIALS AND METHODS

Samples of media were collected from different batches[1] over a period of six months. Two samples of single strength media were collected for each batch appearing in the study. A total of 15 batches was sampled. Each batch was from one of two lots.[2] The samples were stored in a refrigerator until assayed for differences.

Approximately 1.2×10^6 Bacillus subtilis var. niger spores (AAGF) were deposited on each of two stainless steel planchets. The inoculated planchets were conditioned for 18 hours at 22°C, 50%RH and then heated at 125°C for 40 minutes in the clean room using the planchet-boat-hotplate system. Forty minutes of heating gave an expected count of 100 to 200 colonies per plate. The two heated planchets were then insonated for two minutes in one flask containing 100 ml buffered distilled water. One ml of this suspension was plated for each tube of media tested. One operator using one pipette dispensed the 30 aliquots in the test. A completely randomized design was employed. Each of the 30 tubes of media was assigned a random number between one and thirty. Tubes of media were poured serially according to the random number assigned. Incubation was at 32°C for 48 hours.

RESULTS

The plate counts for test BM2305 are listed in Table 3B.1.

[1] All media prepared in our laboratory on the same day from the same bottle of dry media is considered as a single batch.

[2] A lot is a number of batches whose ingredients had the same manufacturer's lot number.

Table 3B.1

Platecounts from Test BM2305, Two Plates Per Batch

Lot Number	Batch Number	Day Batch Prepared	Counts		Lot Means
204691	1	126	135	171	
	2	146	144	147	
	3	152	157	133	
	4	160	168	147	146.125
	5	167	169	157	
	6	180	128	149	
	7	188	136	138	
	8	207	137	122	
205640	9	215	164	156	
	10	276	189	135	
	11	278	146	158	
	12	286	153	157	158.929
	13	292	142	174	
	14	298	159	165	
	15	301	170	157	

The results of an analysis of variance of the data in Table 3B.1 are shown in Table 3B.2. There was no statistical difference found between batches at the 5% level. However, a t-test of the difference in mean counts per lot showed a statistical difference at the 5% level with $t(28) = -2.447$.

Table 3B.2

Analysis of Variance of Plate Count Data for Test BM2305

Source of variation	d.f.	s.s.	m.s.	F
batches	14	3196.2	228.3	.9126
error	15	3752.5	250.2	
total	29	6948.7		
$F(14,15) = .9126 < F_{.95}(14,15)$ Not significant at 5% level.				

A test for trend was made using the non-parametric test for runs.
Each batch was scored + or - depending upon whether or not the observed
average value was above or below the median. The results are shown in
Table 3B.3.

Table 3B.3

Results of Runs Test for Dectecting Trends in Plate Counts

Batch number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Score	−	−	−	+	+	−	−	−	+	+	−	−	+	+	+

Number of runs = 6
Number + = 7
Number − = 8
Prob $(r \leq 6 \mid N_1 = 7, N_2 = 8) = 0.149$

The conclusion based upon the above two-tailed test for runs would
be that there was not enough evidence to conclude that there is a trend
in the average growth inducement potential among batches.

DISCUSSION

If the effect of possible batch differences is constant with respect
to expected $\log_{10}$ count and heat treatment, there would be little if any
effect on the D-value. Intercepts would vary however, depending upon the
lot. If, however, the effect was not constant and varied with the severity
of the heating period, there would be a difference in D-values due to
batch differences.

In view of possible media variation, a good laboratory practice will
be to use media from the same batch to process all samples from one test.
If two batches must be used, it appears that batch differences would be
minimized by using two batches from the same lot that were prepared about
the same time. Media from several batches could be used best for preliminary
or range finding tests.

PROJECT PERSONNEL

The following personnel all made major contributions to this project:
Ronald Jacobson, Bliss Moore, Richard Holcomb, and Yvonne Thun.

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3. LABORATORY CONTROL AND STATISTICAL ANALYSIS

C. SELECTING DILUTION FACTORS WHEN ASSAYING FOR SURVIVORS OF DRY HEAT DESTRUCTION TESTS

Ronald L. Jacobson

The planning of each dry heat destruction test involves anticipating the survivor curve to be obtained. This is necessary in order to select the proper dilutions when assaying for survivors. The following dilution selection chart and table have been used to facilitate the selection of the appropriate dilution factors.

When selecting a dilution plan the expected survivor curve is first plotted on graph paper. The heating times are then selected and the expected numbers of survivors determined.

Using Figure 3C.1 we locate on the graph the expected number of survivors value and then proceed to select that dilution plan that yields an expected plate count between 30 and 300. The interpretation of dilution factors is given in Table 3C.1.

Table 3C.1   Dilution Factors for Different Plans

Plan	Volume (ml)			Dilution Factor		
	Orig susp	Sample to blank	Dilution blank	Amount plated (ml)		
				.1	1	10
1	50	1	50	25500	2550	255
2	50	0	0	500	50	5
3	25	0	0	250	25	2.5
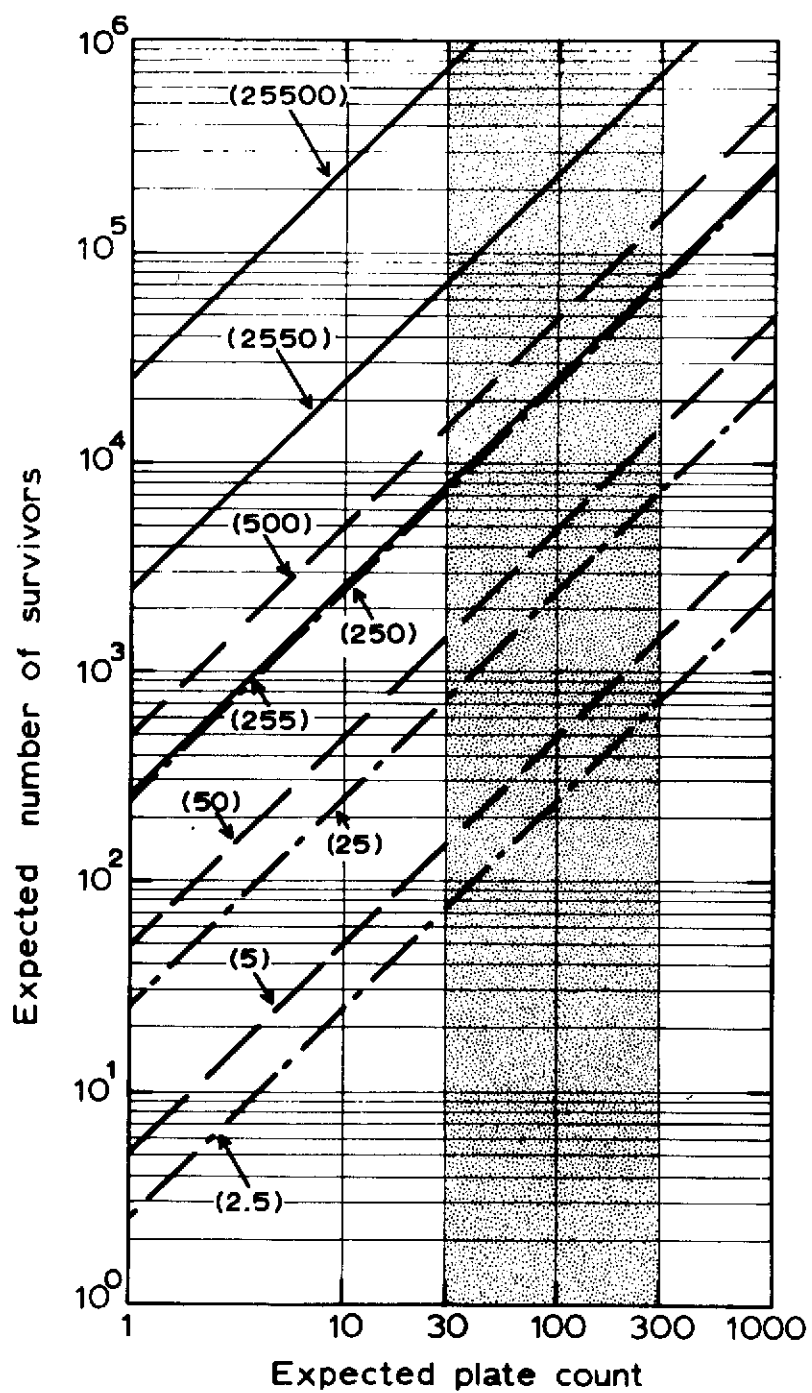
Figure 3C.1: Dilution selection chart. Solid lines are plan 1, dash lines plan 2, and double dash lines plan 3.

3. LABORATORY CONTROL AND STATISTICAL ANALYSIS

D. DEFINITION OF INTERCEPT RATIO (IR)

Richard G. Holcomb, Paul Chapman, Donald Fisher

We have used in our laboratories up to this point a definition of the intercept ratio as follows:

$$IR = \log y_o / \log N_o \qquad (1)$$

where y_o is the intercept of the fitted regression line
and N_o is the initial number of organisms.

The IR values obtained from Y_o and N_o were used to describe the shape of the survivor curves during the first portion of the heating interval. The IR values give the following useful interpretation for most survivor curves:

$IR < 1.0$	survivor plot was curved, opening upward
$IR = 1.0$	plot was linear
$IR > 1.0$	plot was curved, opening downward

The value of IR obtained in equation (1), however, depended intrinsically on the N_o used in the experiments. Two survivor curves that had exactly the same shape would have different IR values if their N_o values were not the same. The IR value was scaled by the N_o value and was not simply a curvature measure of the survivor plot.

We propose a new curvature measure called the Intercept Index (II) which uses only (y_o/N_o). The new index is defined in equation (2):

$$II = \log (y_o/N_o) \qquad (2)$$

The new II value will be an index describing the shape of a survivor curve that will be independent of the curve's position on the log survivor axis. Figure 3D.1 shows two survivor curves plotted on semi-logarithmic

paper. Curve 2 is simply a translation of curve I in the vertical direction by a distance log A.
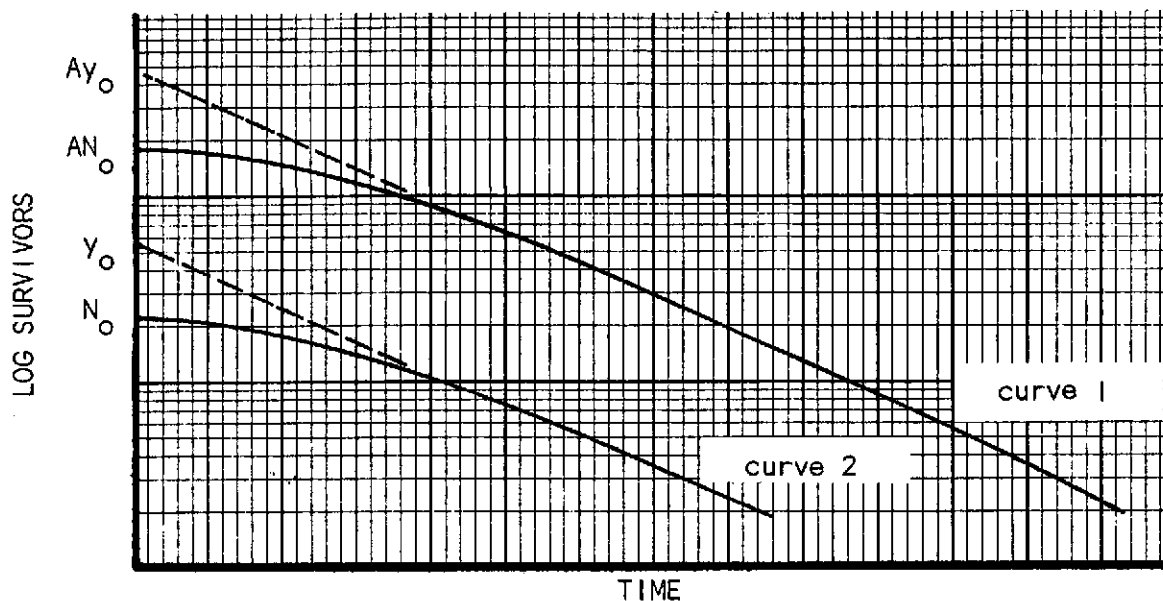
Figure I: Two identically shaped survivor curves.

Old IR values given by equation (I):

$$IR_1 = \frac{\log Ay_o}{\log AN_o}$$

$$IR_2 = \frac{\log y_o}{\log N_o}$$

$IR_1 = IR_2$ only if $A = I$, that is, no translation.

New II values given by equation (2):

$$II_1 = \log (Ay_o/AN_o) = \log (y_o/N_o)$$

$$II_2 = \log (y_o/N_o)$$

$$II_1 = II_2 \text{ for all } A > 0$$

Interpretation of New II Values

Figure 3D.2 shows three y intercepts for hypothetical regression lines fitted to curves 1, 2, and 3, all with the same N_o.
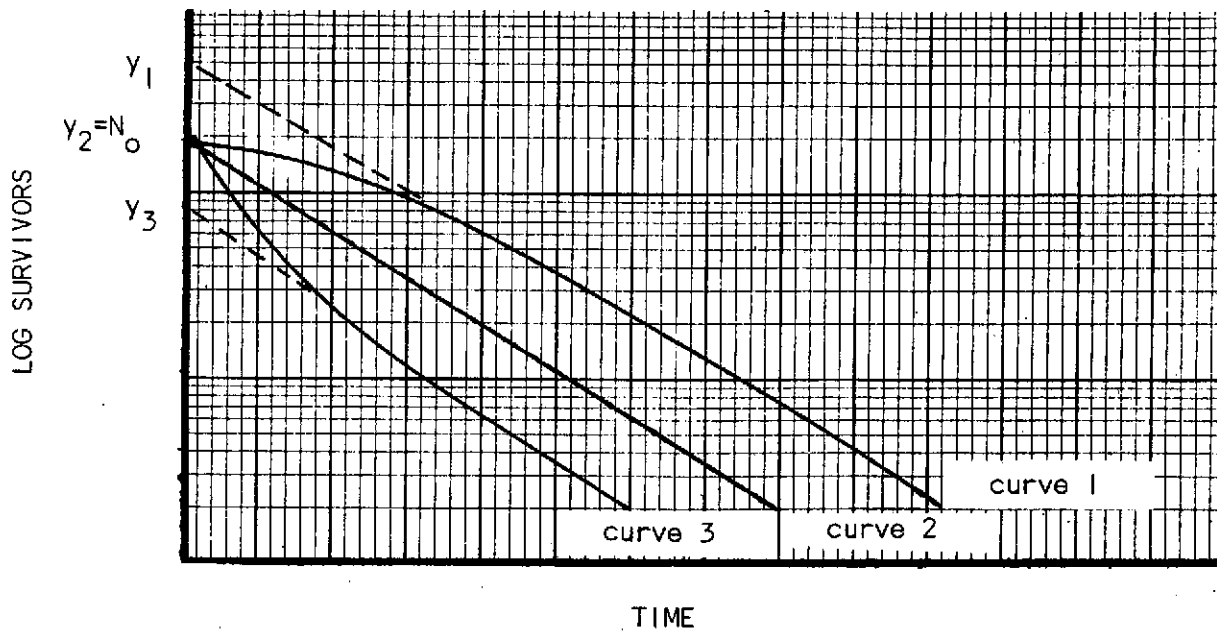
Figure 2: Three survivor curves with the same N_o.

$II_1 = \log (y_1/N_o) > 0$ curve opening downward

$II_2 = \log (y_2/N_o) = 0$ curve essentially linear

$II_3 = \log (y_3/N_o) < 0$ curve opening upward

The table below provides some sample values for the y-intercept and N_o. The IR and II values have been calculated and presented for each pair.

y-intercept	N_o	IR	II
10^4 organisms	10^6 organisms	.667	-2.000
10^5	10^6	.833	-1.000
10^6	10^6	1.000	0.000
10^7	10^6	1.167	1.000
10^8	10^6	1.333	2.000

Table 3D.1:   Sample calculations of IR and **II**

From the table it is seen that a ten fold change in the y-intercept changes the **II** by one unit.

We believe the **II** value can be a very useful tool in characterizing the shapes of survivor curves. Because its value is independent of the position of the survivor curve on the log survivor scale it may be compared with an **II** calculated from another experiment without regard for the N_o values involved.

3. LABORATORY CONTROL AND STATISTICAL ANALYSIS

E. A PROPOSED MODIFICATION OF THE DATA HANDLING SYSTEM

Ronald L. Jacobson

During the early phases of our research efforts in environmental microbiology as related to planetary quarantine, the need for an automated data handling system was recognized. Our first system was presented in Progress Report #4, June 1970, pages 11-22. Some changes and additions to the system were made and reported in Progress Report #6, June 1971, pages 33-40.

The present system provides a data bank of experimental results on punch cards and the software capability of retrieving and presenting these results in a uniform way. However, the data bank does not contain all the data collected in each test. Also, the present system involves the use of several types of data cards that must be punched from specially prepared coding forms. In order to reduce these limitations, modifications of the present system are being considered. One such modification attacks the problem at the data acquisition level.

The combination tabulation and code sheet for plate count data as shown in Figure 3E.1 is being considered to replace the present tabulation sheets and coding forms. The use of the new form would accomplish the following:

A. Eliminate the coding and transferring of data from the original tabulation sheets to coding forms.

B. Improve the ease of preparing punch cards from code sheets by:
1. placing frequently changing information in the early protions of the punch card.
2. reducing the number of card types to two -- data card and comment card.

C. Provide a punch card record of all results obtained in the test.

Figure 3E.1: Tabulation and Code Sheet for Plate Count Data

Tabulation and code sheet for plate count data   1-18-74 RJ

# of lines used _____   Counted by _____   Date _____   Sheet # _____ of _____
          (66-67)   (68-69)   70   71

time units	apparatus	test water content	test temp.	spore code	experiment #
(37)	(41)	(44) ppm	(50) °C	(54)	(59-65)

Line (9) #	Heating (3)+ time	Boat (7) #	Planchet (10) #	Selected (11) LI	Amount Plated (ml)			Dilution factor (31-36) per/ml	Est. Number
					(13) 0.1	(19) 1.0	(25) 10.0		
01									
02									
03									
04									
05									
06									
07									
08									
09									
10									
11									
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34									
35									

One way to use the form is as follows:

A. Complete form

1. Investigator enters codes for the following items and supplies leading zeros as necessary to right justify data in each field (the first column of each field is identified in parenthesis on the form):

   a. heating times

   b. boat and planchet numbers

   c. dilution factor (different factors might be defined in comment cards)

   d. time units

   e. apparatus

   f. test water content and temperature

   g. spore code

   h. experiment number

   i. number of lines used

   j. sheet number

   k. number of sheets used

   l. comments on reverse side of forms (writing in ledgible script should be sufficient)

2. Counter enters:

   a. plate counts

   b. counters initials

   c. date counted

3. Investigator enters:

   a. selected dilution, i.e. .1, 1., 10, or 00 (if no dilution selected for tabulations)

B. Punch cards

1. Keypuncher

   a. duplicates preceding card beginning with column 37 or possibly earlier.

   b. punches comments from reverse side of form onto comment cards by supplying 99 in columns 1 and 2, punching as much of the comment as possible in columns 3-58, the experiment number in columns 59-65, and blanks in columns 66-80 (preparing as many comment cards as necessary).

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C. Verify punched data
    I. Investigator verifies data by
        a. checking entries in tabulation and code sheet with raw data table from computer printout.
        b. marking tabulation and code sheet with information that "data cards were verified."

Implementation of the new form would require a modification of the software by adding a subroutine to the existing program.

However, by using the new form, more information will be available in the data bank; implementation of the new form will make it possible to:

A. Obtain a complete record of the experimental results

B. Conduct more detailed analysis of the data

C. Do future statistical studies in an empirical way, possibly correlating distributional results with dilution factor, amount plated, etc.

Ronald Jacobson and Irving J. Pflug

Dry heat destruction tests of bacterial spores on surfaces were conducted in an environmental chamber shown in Figure AA.I. The chamber, similar to those described in Progress Report #3, pages 53 and 54, was constructed primarily of stainless steel. The front section was equipped with four gloved portholes and a large glass area for observing the interior. An access antechamber was added.

Special peripheral apparatus was installed to allow control of the moisture content of the gas in the environmental chamber. The schematic diagram of Figure AA.2 illustrates the sequence of gas flow through the environmental chamber system. The amount of moisture in the gas was measured by a moisture monitor (Type 26-301, Consolidated Electrodynamics Corporation; Pasadena, California) installed in the gas train as shown in Figure AA.2. Tests were conducted at different controlled levels of moisture in the environmental chamber and monitored between 3 and 1000 parts per million (ppm) by volume.

A dew point analyzer (Model B, Chandler Engineering, Tulsa, Oklahoma,) was used as a check on the moisture monitor (see Figure AA.3). The temperature range of the analyzer was -90°F to 40°F, equivalent to 3.5 to 8200 ppm of moisture.

The moisture monitor data were plotted continuously on a large circular paper chart by an Electronik 15 recorder unit (Honeywell; Fort Washington, Pennsylvania). This equipment was also used to control the operation of the gas conditioner. The gas conditioner apparatus included two silica gel columns, an air pump with electric motor (Dia-pump, Air-Shields, Inc.; Hatboro, Pennsylvania), a solenoid (Automatic Switch Company; Florham Park, New Jersey), and a one-gallon jar of water as shown in Figure AA.4. The gas of the environmental chamber was circulated through the gas conditioner. A continuous flow of the gas was directed through the two silica gel columns. When the solenoid was activated by the moisture controller, a portion of the flow was directed through the water reservoir jar and returned to the chamber.

In Figure AA.5, the chamber pressure regulator and hotplate controllers are pictured. The pressure regulator consisted of a mechanical valve operated by a balanced lever attached to a bellows. The amount of inflation of the bellows was dependent upon the pressure of the gas in the environmental chamber. A positive pressure of about 2.5 inches of water was maintained in the chamber.

Two different temperature controllers were used. The left hotplate was connected to a Model 2156 proportional electronic temperature controller, (Cole-Parmer Instrument Company; Chicago, Illinois). The right hotplate was connected to a Model #7 proportional controller, (RFL Industries, Inc., Boonton, New Jersey).

The access antechamber served as an air lock to receive the transfer trays containing the inoculated planchets on copper boats as shown in Figure AA.6. Use of the antechamber minimized influx of ambient air into the environmental chamber and thus precluded excessive fluctuations in moisture levels. After the gas in the antechamber was purged with gas from the environmental chamber, the transfer trays were moved into the main chamber as shown in Figure AA.7.

The two hotplates measure 10 X 10 X 2 inches. They were made of solid aluminum and encased in a fiberglass insulated box. One box was metal (the right one) and the other was wood. Four 150-watt heaters (Chromalax Type C-206) were located in the hotplates. Also, thermistors and copper-constantan thermocouples were located in the plate. The surface of the plate was polished.

The cold plate measured about 12 X 14 X 1/8 inches. Copper coils were cemented to the underside of the plate. Cold tap water was circulated through the coils.

The thermocouples were connected to a 2012 Data Acquisition System (Hewlett-Packard; Palo Alto, California). The millivolt readings were displayed on the panel and printed (or punched on paper tape).

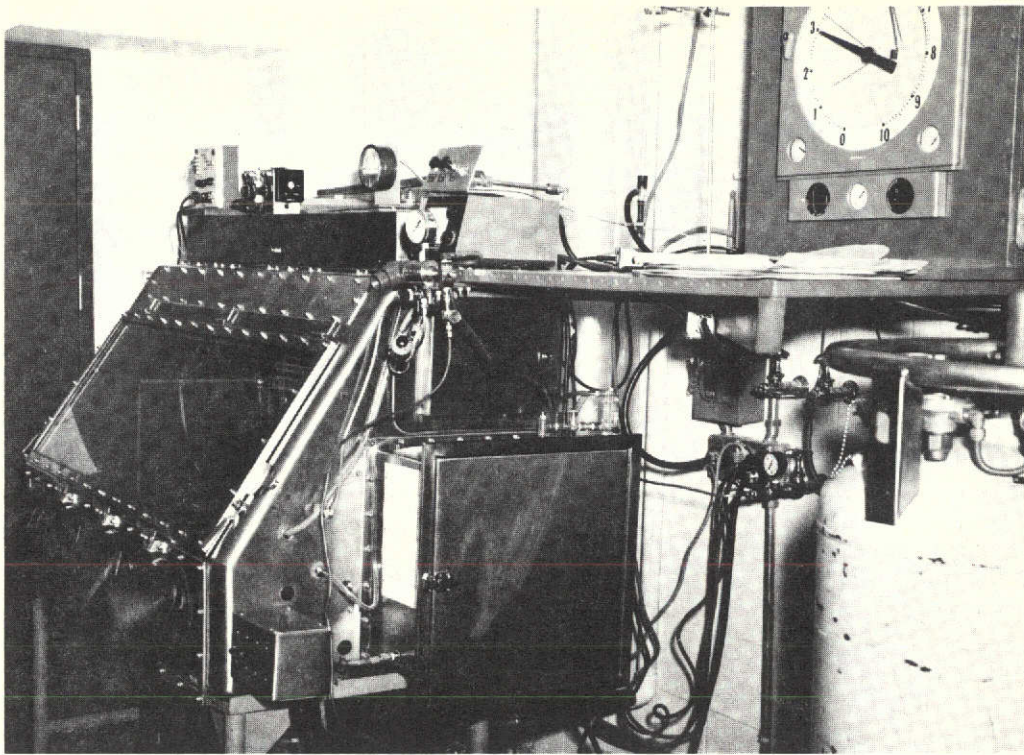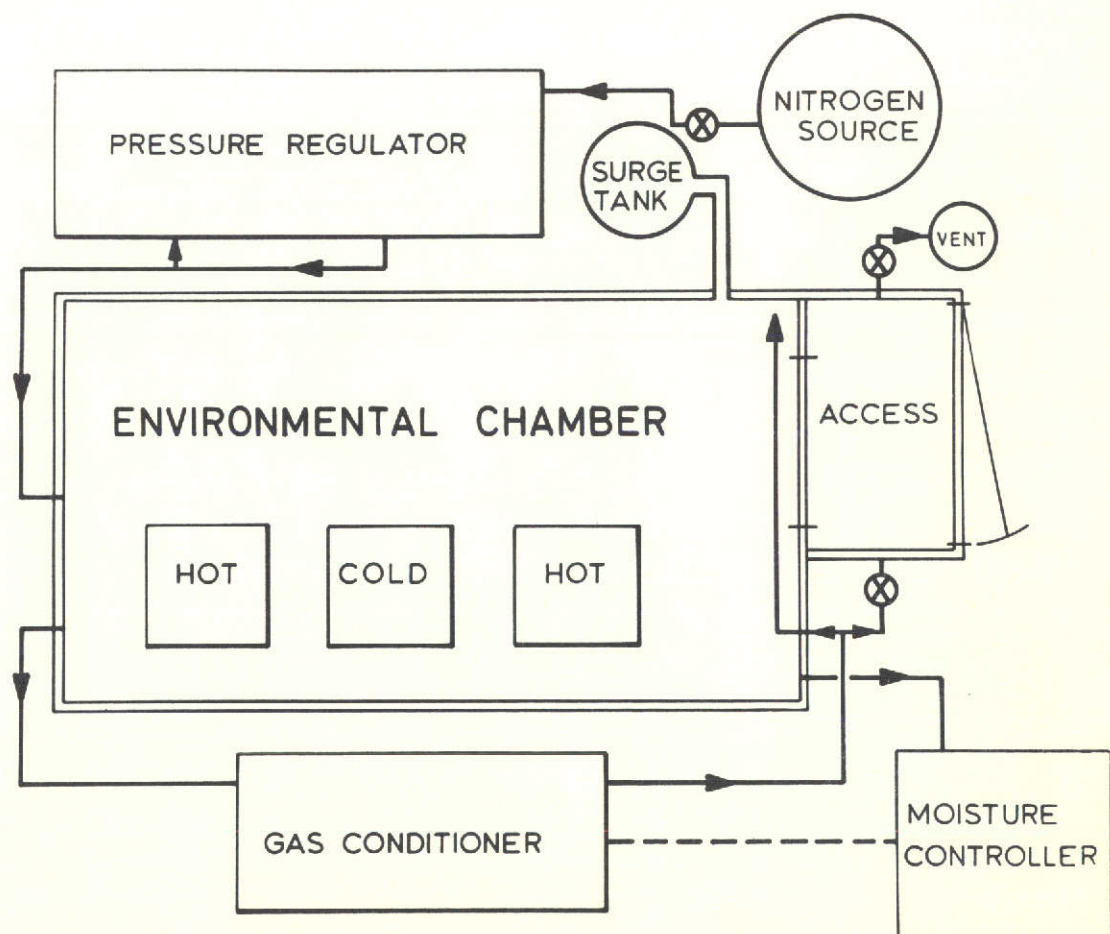Figure AA.I: Environmental chamber and attached equipment.

Figure AA.2: Schematic diagram of environmental chamber system.
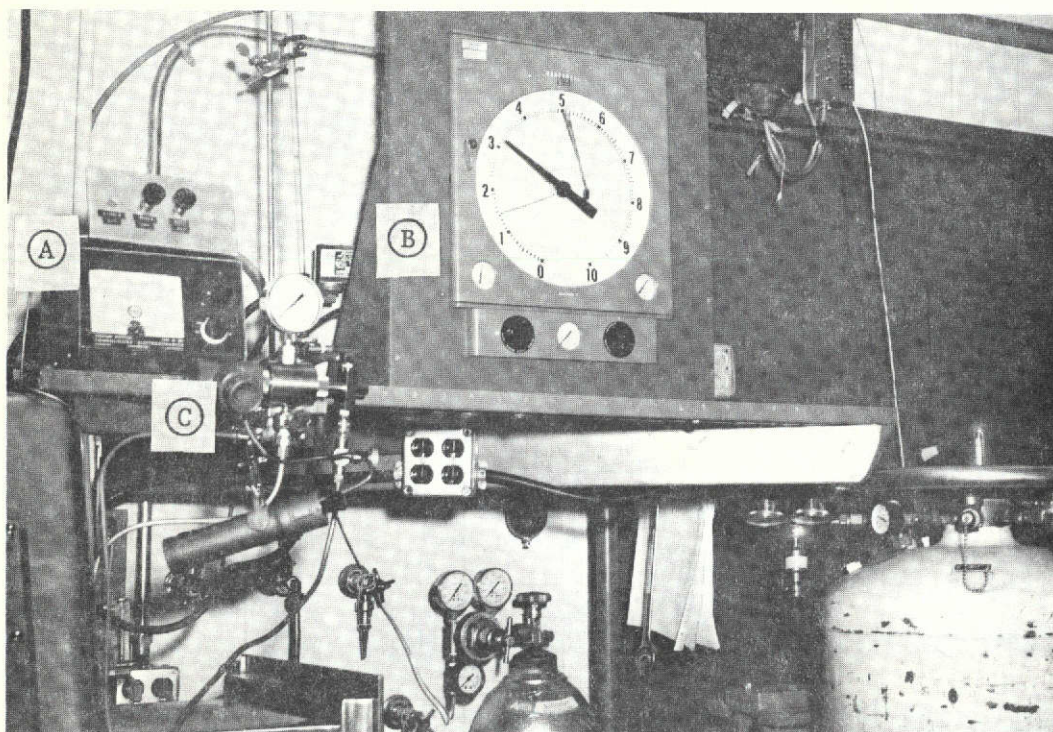
Figure AA.3: Moisture monitor (A), recorder (B) and dew point analyzer (C).
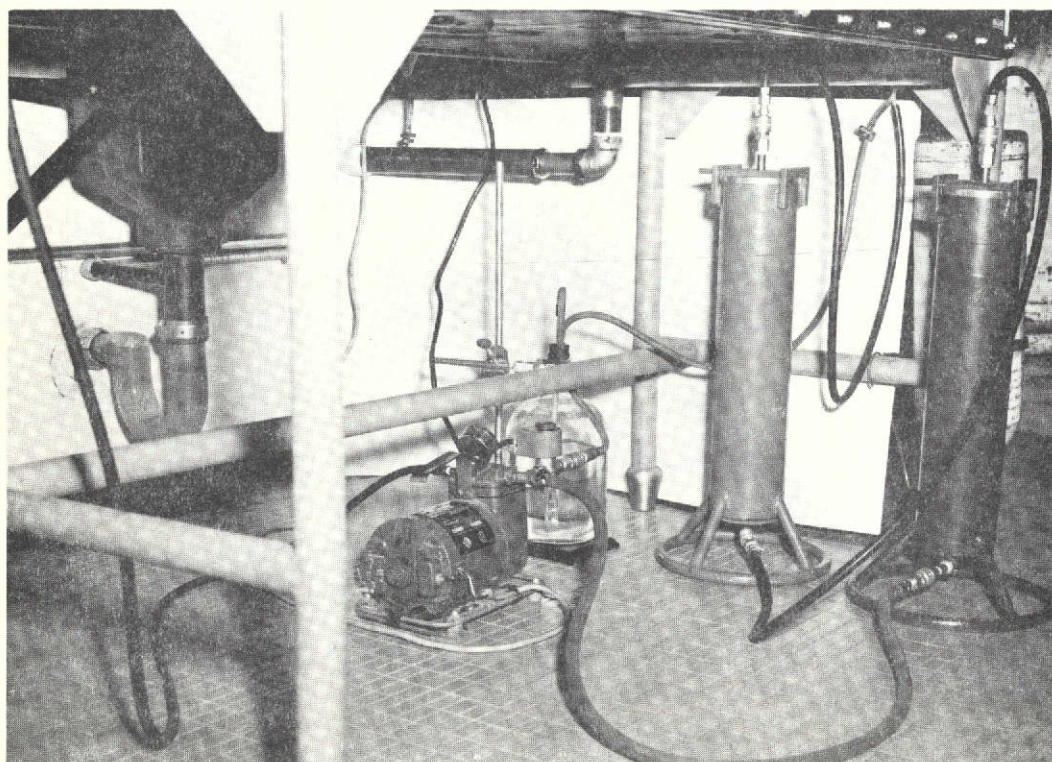
Figure AA.4: Components of gas conditioner.
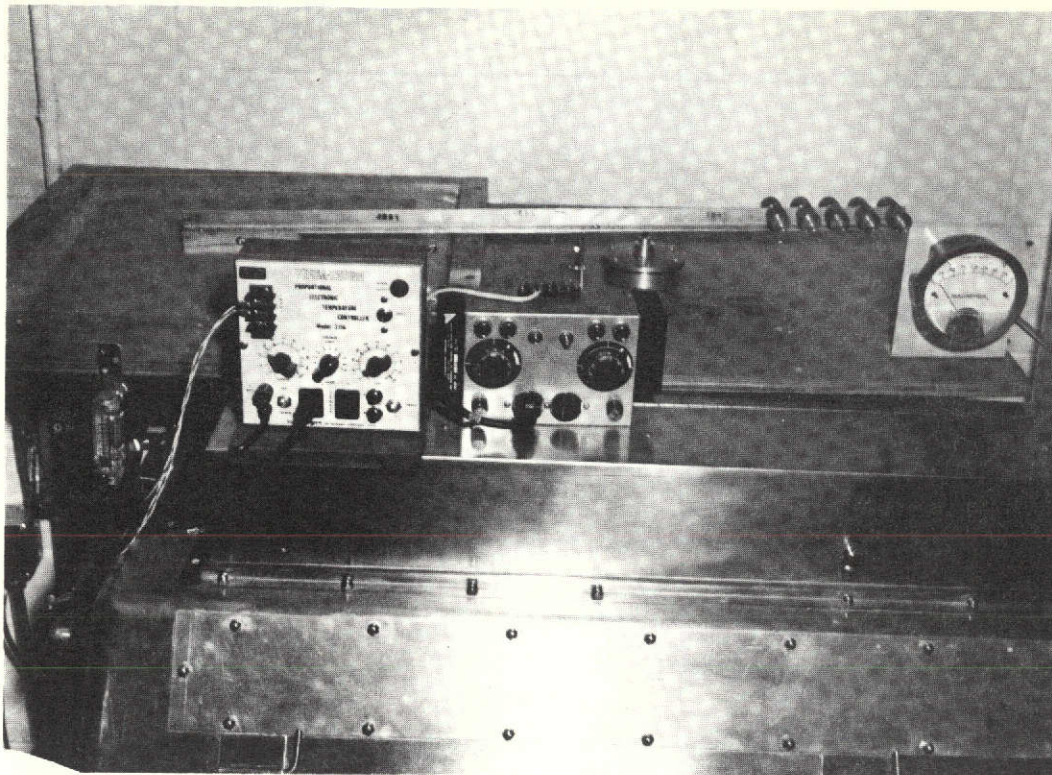
Figure AA.5: Pressure regulator and hotplate controllers.
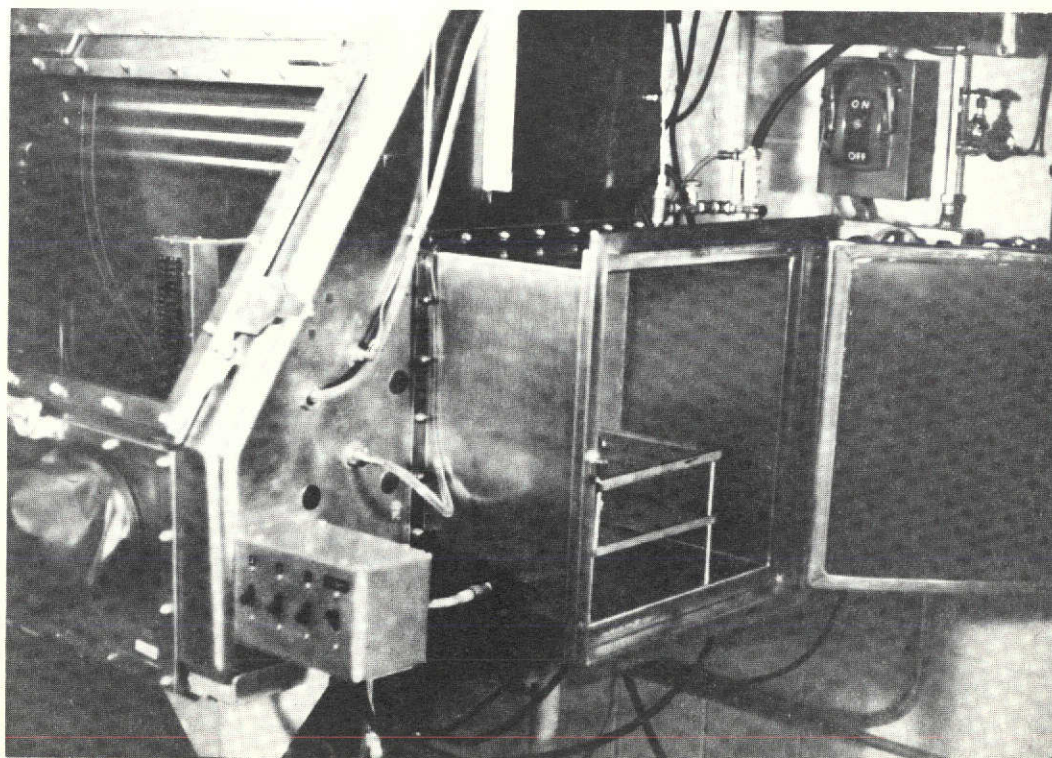
Figure AA.6: Access antechamber of environmental chamber.
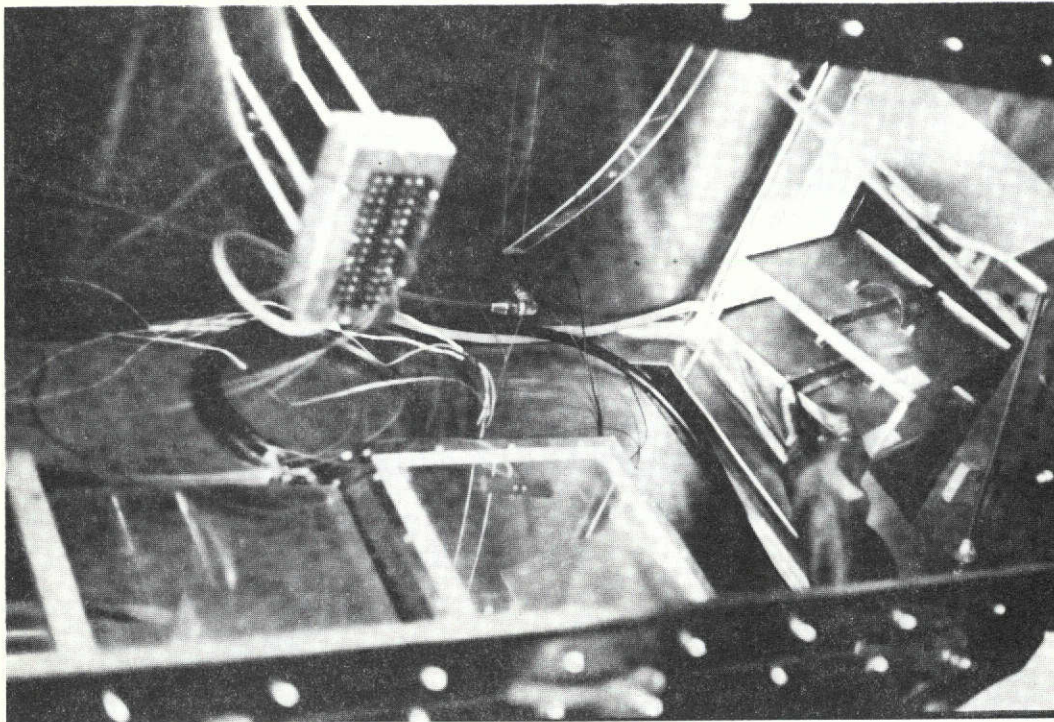
Figure AA.7: Interior of environmental chamber.

APPENDIX B: ADDITIONAL REMARKS ON THE INTER-LABORATORY D-VALUE EXPERIMENTS

Ronald Jacobson

In Progress Report #4, June 1970, pages 57-62, the results of tests on similar organisms at two locations were presented. In conclusion, the different D-values obtained at the two laboratories were attributed to the difference in spores and the different heating methods.

Although it is impossible to specify all the reasons for the different D-values obtained at the two laboratories, the effects of different water contents of the spores at test time and the effect of different heating temperatures might be prime candidates. As discussed in Progress Report #5, December 1970, page 36, the effect of a difference of one degree in the heating temperature is appreciable. Assuming a z-value of 21°C and a $D_{125°C}$-value of 30, the $D_{126°C}$ would equal 33.5 and $D_{124°C}$ would equal 26.8.

Since publication of the original report, it has been observed that the ratio of the D-values obtained for the two different spores were remarkably similar between days (tests). The ratios are listed in Table AB.1.

Table AB.1

Ratios of D-Values Obtained on Different Test Days

Test Date	$D_{125°C}$-value P	M	Ratio P/M
1/13/70	45.52	23.91	1.903
1/14/70	34.77	19.85	1.751
1/27/70	28.96	15.01	1.929
1/28/70	25.79	13.57	1.900

This observation suggests that the relative potency (or lethality) of the Phoenix spore to the Minnesota spore is similar between days even though the D-values differ between days. If we assume that the z-values for the two spores are the same and that the temperatures, for example, differed significantly between days, the similar ratios would be expected.

The use of a comparative assay in the examination of differences between laboratories was discussed by C. I. Bliss (1969).[1] He alludes to a study between laboratories conducted in the early 40's where two samples of digitalis tincture were sent to each lab. Unknown to the laboratory personnel, the two samples were of different concentration; the second sample was an 85% concentration of the first sample.

The results showed a difference in mean just-toxic dose between labs, but a remarkable similarity in the reported relative potencies. He comments that the comparative assay was officially adopted in the next revision of the Pharmacopeia.

The above observations suggest that the type of dry heat destruction data amenable to synthesis between laboratories might be of the relative potency type.

[1] Bliss, C. I. (1969): Communication between biologists and statisticians, a case study, The American Statistician, vol. 23, pp. 15-20.

APPENDIX C: STERILIZATION OF SPACE HARDWARE*   ᴮ

Irving J. Pflug

The purpose of this interim report is to (1) indicate that the reassess-
ment is under way, (2) state that there is still important data in the
process of being gathered that we will want to consider--we must wait with
the main part of this reassessment until these data are available--and
(3) to suggest that we address ourselves now to a consideration of the
form that the sterilization design for space hardware should take in the
future with the idea that the present form may need to be changed.

Before I introduce the discussion of the form of the sterilization
process design, I am going to very briefly review the progress of the
NASA Planetary Quarantine Program in sterilization microbiology and outline
some factors that I believe may serve as a basis for the final sterilization
process design.

A.   Planetary Quarantine Progress in Sterilization Microbiology

I believe it is important that we periodically look back and survey
the trail we have made in conquering the mountain, in this case, under-
standing sterilization processes.   I believe that the NASA Planetary
Quarantine Programs have made a major contribution to the knowledge of
sterilization processes.   This knowledge is, of course, important to NASA
programs, but it is also a real contribution to science in general and
specifically to all industries that must either sterilize products or objects.

At the beginning of the NASA Planetary Quarantine Program, dry-heat
sterilization was thought to be a straight forward process that was a
function only of time and temperature.   There was essentially no knowledge
regarding the role of water in a dry-heat sterilization process, the
magnitude of variation in survival time among different species, and the
variation in survival time between protected and nonprotected organisms.

Research into dry-heat sterilization by persons supported by the NASA
Planetary Quarantine Program has quantitatively established the importance
of water in dry-heat resistance.   In this program it also has been
established that the physical location of organisms, whether they are on
surfaces, in mated surface areas or encapsulated in solids, has an effect
on the dry-heat resistance of the microorganisms.

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*
Report prepared for the Planetary Quarantine Program by I. J. Pflug,
September 24, 1972.

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More recently the program has moved to the point where there has been recognition that we can have micro encapsulation of microorganisms either as a growth phenomenon or as an environmental phenomenon where the resulting particles (encapsulated microorganisms) can be either on surfaces, in mated surface areas, or themselves encapsulated in a piece of hardware.

There are several major implications of these recent developments in dry-heat sterilization. They are:

1. Water is a critical material as far as microbial survival or death is concerned.

2. The degree of protection can be a function of the size of the particle, with larger particles giving greater protection and hence longer survival times than smaller particles.

B. Important Factors in Reassessing the Design Criteria of Space Hardware Sterilization Processes

The need to select a representative microbial inoculum for use in a spacecraft terminal sterilization cycle verification test (STSCVT) has forced us to establish first in our own minds and later physically the quality and quantity of both microorganisms and physical soil that will be on the spacecraft at the time of terminal sterilization.

In facing this challenge there are some pertinent observations that I believe are worth listing which may serve as a basis for discussions that ultimately will lead to the solution of our problem.

1. Microbial spores can remain viable after being subjected to dry-heat conditions for long periods of time. The length of time is a function of species, growth conditions of the spores and the physical and chemical characteristics of the system in which the spores are located during dry-heat treatment. Longest survival times have been reported for organisms native to and still located in soil. Koesterer (Report NASA Contract NASA-550, August 31, 1963) found survivors after heating garden soil for 220 hours at 125°C. Favero (Report 32 and 33 NASA Contract W-13, 062, January 1971 and April 1971) reported survivors from the Cape Kennedy soil after 48 hours at 125°C. In contrast to these large survival times for spores in soil, Bruch (Spacecraft Sterilization Technology, NASA SP-108, 1966) reported that vacuum cleaner dust was sterile after 6 hours at 125°C. Wardle reported (minutes of PQAP meeting, Seattle, 18, 19, 20, 21 June 1971) no viable organisms after heating fallout strips (from Cape Kennedy

AO and ESF facilities) for 13 hours at 125°C. It seems to me the data in the literature appear to be saying, in general, microbial spores in soil are extremely difficult to kill, however, the spores on fallout strips, on hardware, and in dust are relatively easily killed.

2. The spacecraft will be assembled under controlled environmental conditions and periodically will be subjected to a cleaning routine. Therefore, the quality and quantity of soil on the lander is subject to some control. The STSCVT must reflect the conditions that will be found on the flight hardware at the time they are sterilized. If these conditions are not known today, then a range of conditions should be projected and evaluated that will allow for the development of a sterilization cycle for each of the specific assembly conditions: for example, assembly in a laminar flow tent, assembly in a class 10,000 clean room, assembly in a class 100,000 clean room or other assembly conditions.

3. The survival characteristics of a mixed microbial spore population consisting of many different microbial species with variations in heat resistance among species plus variation in their physical state all the way from naked spores to spores that are encapsulated by or clumped together with colloidal or crystalline soil particles certainly do not follow a classical exponential destruction rate pattern or mimic the experimental survival pattern for a homogeneous culture of one of the component organisms. Therefore, as we move in our sterilization cycle design from a pure culture basis to a natural inoculum, I believe that we must change our design criteria from a D-value plus the number of microorganisms basis to a sterilization-time basis.

C. Topics for Consideration and Discussion by PQAP that Will Have an Important Bearing on this Reassessment

Form of the Design Criteria of a Spacecraft Sterilization Process

The precise design of a sterilization cycle requires (1) accurate knowledge of the types and number of microorganisms on the object to be sterilized; (2) the destruction rate characteristics of each type of organism for the conditions that the microorganisms will be subjected to at the time of sterilization; and (3) the temperature coefficient of

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the destruction rate model of these organisms if the sterilization cycle is to be integrated over the heating up and cooling portion of the sterilization cycle. Data are available for Bacillus subtilis var. niger in the form of D-and z-values and the shape of the destruction rate and temperature coefficient curve have been established. To obtain the same data for all possible organisms that will be found in natural soils is a task that will require a long period of time and a lot of financial resources. Therefore, it seems to me that a decision will have to be made regarding either the use of (1) Bacillus subtilis var. niger for sterilization cycle design or (2) the utilization of the natural flora found in a typical spacecraft assembly area.

I suggest that if we now proceed to use a natural flora in tests to determine the design criteria for terminal sterilization of the space- craft that we change our design criteria from a D-value plus the number of microorganisms basis to a sterilization time at a temperature for a specific assembly environmental condition basis. To obtain these data will require replicate tests under different inoculum conditions (dis- cussed in the following section, "Evaluation of Natural Inoculums") and at several different temperatures if the terminal sterilization cycle is to be integrated over a range of temperatures (it will be necessary to develop a thermal death time, TDT, curve for each condition).

There are a number of reasons for considering a change from the D-value concept to the TDT curve concept. However, the immediate reason is that in a natural inoculum the destruction model (shape of the survivor curve) is not known and will be difficult to establish and characterize.

Evaluation of Natural Inoculums

If we are going to base our spacecraft Sterilization Process design on data obtained from the sterilization characteristics of a simulated lander located in a typical assembly area, then I believe it is important that we not only have a very good program for determining the thermal death time curve for a simulated lander in a single environment but that we also evaluate a range of environments; I suggest that at least three and perhaps as many as five environments covering the range of conditions be evaluated. It seems to me that the experimental conditions should extend from a class 100-type clean room or clean tent on one extreme to an open-bay area at the other end of the range--all of these conditions being located in the Cape Kennedy area.

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I believe that it is desirable that we have thermal death time curves for all conditions. If this is not possible, then we should have a TDT curve for the environmental condition most likely to exist in the spacecraft assembly area and an F_{125} value only for the other condition.

I believe that before we proceed with this program there should be a systems analysis made of the TDT data program so that we are sure that we have adequate background knowledge before we plunge into this new program of data gathering. It is important that we duplicate as well as possible the conditions that the potential spacecraft will be assembled under in each of these conditions so that our TDT data are realistic. This is more critical with the TDT type of system than with the D-value, number of organisms system. It is important that we have both statistical input and engineering input as well as microbiological input in planning this program.

APPENDIX D: DECIMAL REDUCTION TIME ("D VALUES") ESTIMATION IN THERMAL MICROBIAL STERILIZATION (A PRELIMINARY REPORT)

R. B. McHugh and N. Sundararaj[*]

INTRODUCTION

Sterilization of microbial populations, particularly by heat, retains a position of great importance in a variety of areas: medicine, the food industry, sanitary engineering, and planetary quarantine among others. Assessing the efficacy of sterilization technique is, therefore, a recurring problem in test design and analysis.

Attention is confined here to heat as the mode of sterilization, as applied for example in the dry-heat sterilization of bacterial spores on planetary lander capsules.[1] Current research of Pflug[2] and associates will be used for illustration.

The rate of thermal sterilization of a microbial population is exponential in form in a wide variety of situations.[3] Restricting the present study to such cases, the number of surviving cells $N(t)$ after exposure for time t is given by

$$N(t) = N_o \cdot 10^{-Kt} \qquad (1)$$

Here N_o is the initial size $N(0)$ of the population of viable bacteria. The constant K is the risk of death for any organism, reflecting the sensitivity of the strain and the intensity of the thermal agent. The larger K, the faster the rate of kill. Inversely proportional to K is the more commonly used index, D. The "decimal reduction-time," D, is defined as the time--for a specific temperature--required to reduce the microbial population by 90 percent. From equation (1), it follows that

$$D \doteq \frac{1}{K} \qquad (2)$$

Hence a convenient form of the theoretical survivor curve of microorganisms

* This report prepared by R. B. McHugh, Professor, and N. Sundararaj, Research Assistant in the Division of Biometry, School of Public Health, University of Minnesota, Minneapolis, Minnesota.

exposed to heat is

$$N(t) = N_o \cdot 10^{-t/D} \tag{3}$$

No difficulties would arise in assessing the efficacy of a technique of thermal microbial sterilization if N_o and $N(t)$ at a given time point were known without error. In this event, equation (3) could be solved for D as

$$D = \frac{t}{\log N_o - \log N(t)} \tag{4}$$

In practice, however, the population quantities N_o and $N(t)$ are unknown. What can be obtained are sample observations $n(t_h)$ of $N(t_h)$. The estimates $n(t_h)$ of the number of organisms surviving the heat treatment at times t_h (h = 0, 1, ... H) are arrived at by colony count.[3]

The biometric problem then arises: How should the $n(t_h)$ best be employed to estimate N_o and D; and how can the statistical uncertainty in the resulting estimators $\hat{N}_o$ and $\hat{D}$ be measured? In addition, various design problems should be considered: for acceptable levels of uncertainty and cost, how many time points H are needed and how should they be spaced; how many replicate units (planchets, see below) should be heated and how many replicate plates poured for the colony count?

Attention should also be given to the problem of comparing several D-values, from each of several populations corresponding to different experimental treatments.

The data[4] in Table AD.I (see page 86) are illustrative. Twenty μl of an aqueous spore suspension of _Bacillus subtilis_ var. _niger_ (approximately 1 X 10^6 spores) was deposited onto each of three sterile, stainless strips (planchets), each 1/2" square, lying flat in a shallow groove running the length of a rectangular copper boat. Seven such copper boats were used, assigned randomly either to no heat or to one of the six heating times. After the application of heat, dilutions were made, plated, colonies were counted, and the number of surviving spores $n(t_h)$ estimated.[2] A point critical to the following discussion is the fact that the counts $n(t_h)$ have a Poisson distribution[4] with mean $N(t_h)$.

THE ESTIMATION OF D

I. A Direct Approach to the Estimation of D

Equation (4) suggests a direct method of estimating the parameter D. Replacing the unknown N_o by n_o (i.e. $n(t_o)$) and $N(t)$ by $n(t_h)$ yields H estimates of D ($h = 1, \cdots, H$). Averaging these H estimates gives

$$\hat{D}_1 = \frac{1}{H} \sum_{h=1}^{H} \frac{t_h}{\log n_o - \log n(t_h)} \tag{5}$$

The precision of D_1 by the propagation of error method[5] and employing the Poisson property that the variance of the $n(t_h)$ is equal to the mean, $N(t_h)$, is:

$$Var(\hat{D}_1) = \frac{(\log_{10} e)^2}{H^2} \sum_{h=1}^{H} t_h^2 \left[\frac{\frac{1}{N_o} + \frac{1}{N(t_h)}}{[\log N_o - \log N(t_h)]^4} \right] \tag{6}$$

In (6), as in the other variance expressions given below, first order approximations are employed.

2. Another Direct Method for Estimating D

The possible vulnerability of $\hat{D}_1$ to systematic error through an aberrant value of n_o prompts a search for alternatives. An estimator free of n_o and only slightly less direct than (5) is

$$\hat{D}_2 = \frac{1}{H} \sum_{h=1}^{H} \frac{t_{h+1} - t_h}{\log n(t_h) - \log n(t_{h+1})} \tag{7}$$

The basis for (7) is the logarithmic form of (3), viz.,

$$\log N(t) = \log N_o - \frac{t}{D} \tag{8}$$

Differencing (8) for two successive t values, t_h, t_{h+1}, eliminates log N_o and yields

$$\log N(t_h) - \log N(t_{h+1}) = \frac{t_{h+1}}{D} - \frac{t_h}{D} \quad .$$

Solving this expression for D, replacing $N(t)$ by $n(t)$, and averaging over h yields (7).

This alternative approach, applied to the reciprocal of D(cf eq (2) was in early use by Chick.[6] She did not, however, state the precision which, by the "Poisson property" and the propagation of error, is

$$\text{Var}(\hat{D}_2) \triangleq \frac{(\log_{10} e)^2}{H^2} \sum_{h=1}^{H} (t_{h+1} - t_h)^2 \left[\frac{\frac{1}{N(t_h)} + \frac{1}{N(t_{h+1})}}{\left[\log N(t_h) - \log N(t_{h+1}) \right]^4} \right] \quad (9)$$

3. The Quasi Least Squares Estimation of D

A current[1] estimation procedure that appears to be of frequent use is

$$\hat{D}_3 = - \frac{\sum\limits_{h=1}^{H} (t_h - \bar{t})^2}{\sum\limits_{h=1}^{H} (t_h - \bar{t}) \log n(t_h)} \quad (10)$$

The rationale for (10) also is based upon a property of the logarithmic form (8), viz., the fact that log $N(t)$ is a linear function of t, say

$$Y(t) = \alpha + \beta t \quad (11)$$

where $\alpha = \log N_o$, and $\beta = -\frac{1}{D}$. $\quad (11')$

Replacing $Y(t) = \log N(t)$ by the H observations $y(t_h) = \log n(t_h)$ for $h = 1, \cdots, H$, the estimator $\hat{D}_3$ is obtained from the minimization of

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$$\sum_{h=1}^{H} \left[\log n(t_h) - \log N_o - t_h / D \right]^2 \tag{12}$$

with respect to $\log N_o$ and $-\frac{1}{D}$.

The minimization in (12) gives a least squares fit of $y(t_h)$ and t_h to the model (11). That is, it gives a least squares fit of $\log n(t_h)$ and t_h to the model (8). Hence $\hat{\beta} = (-\frac{1}{\hat{D}_3})$ is a least squares estimator of $\beta = -\frac{1}{D}$, but $\hat{D}_3 = -\frac{1}{\hat{\beta}}$ is not a least squares estimator of D . For this reason, $\hat{D}_3$ is here termed the "quasi least squares estimator" of D. The actual least squares estimator of D is given by $\hat{D}_4$ below.

The precision of $\hat{D}_3$ is

$$Var(\hat{D}_3) = \left[\frac{\displaystyle\sum_{h=1}^{H} \left\{ (t_h - \bar{t})^2 \ \frac{1}{N(t_h)} \right\}}{\left\{ \displaystyle\sum_{h=1}^{H} (t_h - \bar{t})^2 \right\}^2} \right] D^4 \tag{13}$$

4. The Least Squares Estimation of D

Because the counts $n(t_h)$ have a Poisson distribution with mean $N(t_h)$, the residuals $n(t_h) - N(t_h)$ have a Poisson distribution with mean zero. Denoting the residuals by ε_h and using (3) , the basic statistical model throughout the discussion is

$$n(t_h) = N_o \cdot 10^{-t_h/D} + \varepsilon_h \tag{14}$$

where $E(\varepsilon_h) = 0$ and $Var(\varepsilon_h) = N(t_h)$.

The least squares estimator of D, say $\hat{D}_4$, is therefore the solution for D which minimizes

$$\sum_{h=1}^{H} \varepsilon_h^2 = \sum_{h=1}^{H} \left[n(t_h) - N_o \cdot 10^{-t_h/D} \right]^2 \qquad (15)$$

with respect to N_o and D. The solution is obtained iteratively.

The precision of $\hat{D}_4$ is also obtained iteratively after first expanding[8] $N_o 10^{-t_h/D}$ in a Taylor series about initial values of N_o and D..

5. The Weighted Least Squares Estimation of D

It is clear that the errors ε_h in the model (14) do not have homogeneous variance. That is, $Var(\varepsilon_h) = N_o \cdot 10^{-t_h/D}$ is a function of t_h (for h = 1 $\cdots\cdot$ H). Because of this variance heterogeneity, weighted least squares estimation of D may be expected to be more nearly optimum, in the sense of greater precision, than is the ordinary least squares estimation procedure of equation (15).

Employing as weights the reciprocals of the variances of the ε_h , the weighted least squares estimator of D, say $\hat{D}_5$, is the solution for D which minimizes

$$\sum_{h=1}^{H} \frac{1}{N(t_h)} \varepsilon_h^2 = \sum_{h=1}^{H} \frac{10^{t_h/D}}{N_o} \left[n(t_h) - N_o 10^{-t_h/D} \right]^2 \qquad (16)$$

with respect to N_o and D.

The solution to (16) as well as the precision of $\hat{D}_5$, are obtained iteratively.

6. The Maximum Likelihood Estimation of D

The most complete utilization of the fact that the $n(t_h)$ have a Poisson distribution is to employ the joint probability distribution of the $n(t_h)$ to obtain the maximum likelihood estimator of D. This estimator is that solution, say $\hat{D}_6$, for D which maximizes with respect to N_o and D

$$\sum_{h=1}^{H} \log \left\{ \frac{\left[e^{-\left(N_o \, 10^{-t_h/D}\right)} \right] \left[N_o \, 10^{-t_h/D} \right]^{n(t_h)}}{[n(t_h)]!} \right\} \qquad (17)$$

The maximum likelihood equations are solved iteratively.

The precision of $\hat{D}_6$ is

$$\text{Var}(\hat{D}_6) = \frac{\displaystyle\sum_{h=1}^{H} 10^{-t_h/D}}{N_o \cdot I} \qquad (18)$$

where

$$I = \left[\frac{\ell n \, 10}{D^2} \right]^2 \left\{ \left[\sum_{h=1}^{H} t_h^2 \, 10^{-t_h/D} \right] \cdot \left[\frac{\displaystyle\sum_{h=1}^{H} 10^{-t_h/D}}{N_o} \right] - \left[\sum_{h=1}^{H} t_h \, 10^{-t_h/D} \right]^2 \right\} \quad (19)$$

Note: The foregoing six methods yield approximate $(1 - \alpha)\,\%$ confidence limite for D according to

$$\hat{D}_m \pm z_{1-\alpha/2} \, SE(\hat{D}_m) \qquad (20)$$

(for m = 1 , 2 , $\cdots$ 6).

Here $z_{1-\alpha/2}$ is the standard normal deviate at the $1 - \alpha/2$ percentile point. The quantity $SE(\hat{D}_m)$ is the square root of the estimate of precision of $\hat{D}_m$, an estimate obtained by replacing the unknown quantities in each $\text{Var}(\hat{D}_m)$ by the corresponding sample estimates. Confidence limits on D were also obtained by the two additional procedures outlined below.

7. Interval Estimation of D from β : Poisson Precision

This procedure, an alternative to the quasi least squares interval estimator $\hat{D}_3 \pm z_{1-\alpha/2} \ SE(\hat{D}_3)$, has two steps, based on the relation (11') between D and β.

(a).   Limits are first obtained on the β of (11), using $\hat{\beta}$, the negative reciprocal of (10), as

$$\hat{\beta} \pm z_{1-\alpha/2} \ SE(\hat{\beta}) \tag{21}$$

The $SE(\hat{\beta})$ used above is the square root of

$$Var(\hat{\beta}) = \frac{\sum_{h=1}^{H} \left[(t_h - \bar{t})^2 \cdot \frac{1}{N(t_h)} \right]}{\left[\sum_{h=1}^{H} (t_h - \bar{t})^2 \right]^2} \tag{22}$$

upon replacing $N(t_h)$ in (22) by $n(t_h)$. The precision formula (22) for $\hat{\beta}$ is derived by propagation of error and the Poisson property.

(b).   The second step consists of obtaining the negative reciprocals of the limits on β in order to arrive at a confidence interval for D. This results in

$$\left[- \frac{1}{\hat{\beta} - z_{1-\alpha/2} \ SE(\hat{\beta})} \ , \ - \frac{1}{\hat{\beta} + z_{1-\alpha/2} \ SE(\hat{\beta})} \right] \tag{23}$$

as limits on D .

8. Interval Estimation of D from β : Quasi Least Squares Precision

This procedure, also an alternative to $\hat{D}_3 \pm z_{1-\alpha/2} \ SE(\hat{D}_3)$, has the same two steps of 7. above except that the $SE(\beta)$ used in (21) is different. Here the customary formula for $SE(\hat{\beta})$ from standard linear regression theory is used, as suggested by the model

$$y(t_h) = \alpha + \beta\, t_h + \varepsilon \qquad\qquad (24)$$

where α and β are given in $(11')$ and $y(t_h) = \log n(t_h)$.
This customary formula for $SE(\hat{\beta})$ is then

$$\left[\frac{\displaystyle\sum_{h=1}^{H} \left[y(t_h) - \hat{\alpha} - \hat{\beta}\, t_h \right]^2 \Big/ (H-2)}{\displaystyle\sum_{h=1}^{H} (t_h - \bar{t}\,)^2} \right]^{1/2} \qquad\qquad (25)$$

where $\hat{\alpha} = \bar{y}(t) - \hat{\beta}\,\bar{t}$

Example

The above six procedures for the point and interval estimation of D, together with the two additional procedures for the interval estimation of D, have been applied to the data of Table AD.1. The results are presented in Table AD.2.

Table AD.1

The Estimated Number of Spores* in Original 50 ml Suspension at the Times Listed in Column 2, with Two Determinations for Each Planchet and with 3 Planchets for Each Boat.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Boat No.	Time in Min.	Planchet 1 Determination No.		Planchet 2 Determination No.		Planchet 3 Determination No.		Arith. Mean
		(1)	(2)	(1)	(2)	(1)	(2)	
1	0	1275000	1504500	1224000	1504500	1657500	1504500	1445000
2	75	90525	112200	119850	104550	124950	109650	110288
3	75	144075	116025	112200	112200	93075	85425	110550
4	150	18800	22200	20800	21400	16600	15900	19283
5	225	7975	7925	7625	6700	6875	7450	7425
6	300	700	825	975	950	750	475	779
7	300	1050	850	1150	1150	1000	775	996

*
Bacillus subtilis var. niger. Samples were conditioned at 23°C and 39%RH while treated at 100°C and 0.02%RH for times listed. Apparatus Type-Open, Name-Hotplate in Glove Box.

Table AD.2

The Estimates $\hat{D}_m$ (m = 1,...,6) along with Their Estimated Variances, SE's, and 95% Confidence Limits* for the Data of Table AD.I.

Estimator	Point Estimate $\hat{D}_m$	Var($\hat{D}_m$)	SE($\hat{D}_m$)	Confidence Limits on D Interval		
				Lower	Upper	Width
$\hat{D}_1$	84.5655	0.01612	0.126971	84.3116	84.8194	0.5078
$\hat{D}_2$	90.6411**	0.5469	0.7395	89.1621	92.1201	2.9580
$\hat{D}_3$	109.4148	1.3327	1.1544	107.1059	111.7236	4.6177
$\hat{D}_4$	103.2004					
$\hat{D}_5$	113.5034					
$\hat{D}_6$	111.6211	.0113	.3067	111.0077	112.2345	1.2268
Method 7***				107.1536	111.7734	4.6198
Method 8***				98.3727	123.2491	24.8764

* By using normal approx with $\hat{D} \pm 2SE(\hat{D})$

** In case of multiple observations at any t_h, the average value for $n(t_h)$ is used in formulas (7) and (9), with corresponding modification in H.

*** Methods 7 and 8 give confidence intervals for D. They do not yield explicit formulas for $\hat{D}$, Var $\hat{D}$ or SE($\hat{D}$). However, "effective" values of these three quantities, say $\hat{D}^*$, Var($\hat{D}^*$) and SE($\hat{D}^*$), can be obtained from the lower ℓ, and the upper u, confidence limits on D as follows:

$$\hat{D}^* = \ell + \frac{u-\ell}{2} \qquad Var(\hat{D}^*) = \left[\frac{u-\ell}{4} \right]^2 \qquad SE(\hat{D}^*) = \left[\frac{u-\ell}{4} \right]$$

thus

$$\hat{D}_7^* = 109.4635 \qquad Var(\hat{D}_7^*) = 2.4025 \qquad SE(\hat{D}_7^*) = 1.1550$$

and

$$\hat{D}_8^* = 109.4148 \qquad Var(\hat{D}_8^*) = 38.6772 \qquad SE(\hat{D}_8^*) = 6.2191$$

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Irving J. Pflug and Jacob E. Bearman

Sterilization is an interdisciplinary applied science area that draws on biology, mathematics, and engineering for its scientific basis.   In the past mathematicians and engineers have learned a great deal about microbiology from the microbiologists.   Perhaps, when it comes to the analysis of sterilization data, we engineers and mathematicians can make a major contribution to sterilization data analysis and indirectly to the microbiologists.

It has become common place to use certain parameters and terms in the analysis and interpretation of research data on the effect of heat or chemical stress on microbial survival. We believe that these parameters and terms need to be defined at regular intervals to insure that they have a singular meaning and that this meaning is known. We are going to define in detail some of the terms and parameters in use today.   In addition, we are going to suggest when these terms and parameters should be used and some limits that should be placed on their usage.

The survivor curve

In an experiment to evaluate the effect of a heat stress on microbial spores, all conditions except the heating time are usually held as constant as possible. If we subject 10^5 spores per planchet to a dry heat stress at 125°C for 0, 20, 40, 60, 80, and 100 minutes and then enumerate the survivors using the plate count technique, the resulting data will be the number of colony-forming units for each heating time and/or the unheated control.   To communicate these results visually, a survivor curve graph can be prepared where the number of survivors is shown as a function of the length of heating time at temperature T.   A survivor curve graph, where both the number of survivors and time are plotted on an arithmetic scale is shown in Figure AE.1.

In Figure AE.2 a survivor curve graph is shown where the number of survivors has been plotted on a logarithmic scale as a function of time on the arithmetic scale.

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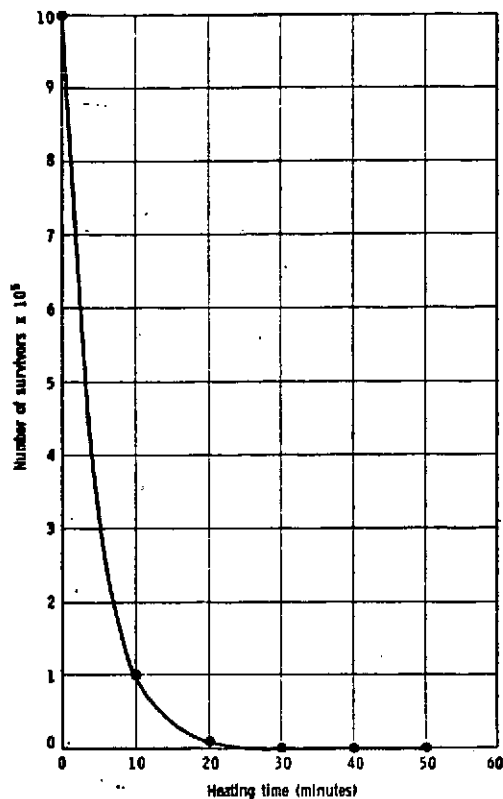
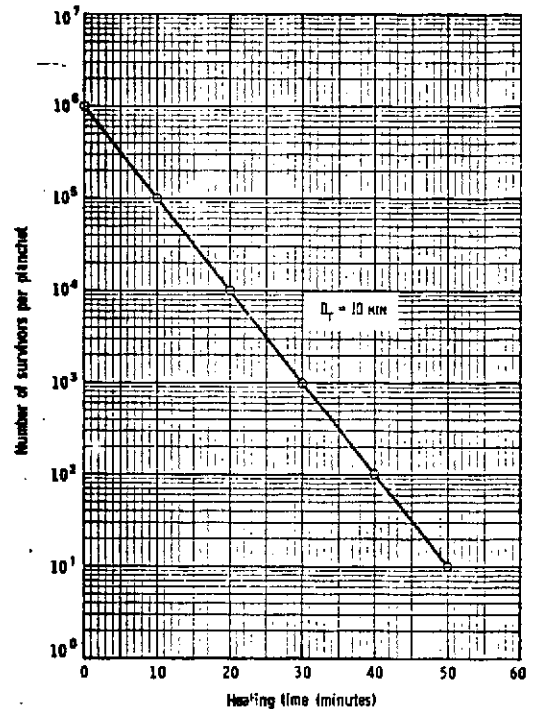Figure AE.1: Survivor curve plotted on arithmetic coordinates.

Figure AE.2: Survivor curve where number of survivors are plotted on a logarithmic scale and time on an arithmetic scale.

The question is asked, "In preparing a graph of the numbers of surviving microorganisms as a function of the severity of the heat or other stress, should we plot the number of surviving organisms or the logarithm of the number of surviving organisms versus time?" In most experiments with which we will be dealing, initial microbial populations are of the order of 10^6 or 10^7 spores per unit, with the final number of survivors being as low as 10 or 100 per unit. If we use an arithmetic scale to cover the range from 0 to 10,000,000 (10^7), the smallest change in numbers we can show graphically will be about 100,000 (10^5). The resulting graph will show only those changes which occur between 10^7 and 10^5. In sterilization studies we are very interested in the rate of destruction of microorganisms as we approach zero survivors; therefore, an arithmetic plot is in general of very little value. A plot of the logarithm of the number of survivors versus time allows us to observe changes in the number of survivors as we approach zero survivors and is therefore a better method for presenting the data regardless of the resulting shape of the survivor curve.

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Experience over a 60-year time period has indicated that when the logarithm of the number of survivors is plotted as a function of time arithmetically (referred to as a semi-log survivor curve) usually part, or all, of the survivor curve is a straight line, or the survivor curve can be represented by two straight lines. However, not all semi-log survivor curves are single straight lines nor can they be described by two straight lines. There are many examples in the literature where the semi-log survivor curve for heat, or other lethal stress, is parabolic or sigmoidal in shape.

D-value

Microbiology is an experimental science. Experiments must be carried out to determine the response of microorganisms to untested conditions. Therefore, uncovering relationships concerning microbiological data is very important. Perhaps this is best accomplished through the use of the semi-log survivor curve. The D-value came into use because it was a simple and convenient way of describing the slope of the semi-log survivor curve that can be approximated by a straight line. The D-value is the time of heating (or other stress) necessary to produce a 90% reduction in the microbial population. In this manner, it is the negative reciprocal of the geometrical slope of the survivor curve.

We suggest that emphasis be placed on the basic definition of the D-value: D-value is the negative reciprocal of the slope of the straight line approximation to the graph of the logarithm of the number of surviving organisms versus time. It is measured as the time required for the number of survivors to be reduced by a factor of 10. One cycle on the logarithmic scale represents a ten-fold change in the number of survivors; therefore, the D-value is the time for the straight line to traverse one logarithmic cycle. When preparing survivor curve graphs, semi-logarithmic graph paper is most convenient to use since the number of survivors can be plotted directly on the logarithmic scale versus time on the arithmetic scale.

Use and abuse of the D-value

The D-value was developed as an aid for relating survivor curve data to time, when the relationship approximated a straight line. However, over a period of more than two decades, its use has changed from being a unit of measure to that of a measure in itself. Many scientists who have recently entered the area of sterilization microbiology use the D-value

as an institution of its own and ascribe to it properties and responsi-
bilities that were neither originally intended nor have recently been
established. Misuse or abuse of the D-value led from the original
relationship concept to an institutional type of attributes which now
in turn are being questioned. In fact, some microbiologists are even
saying that to use the D-value is bad.

Is the D-value a poor tool for describing microbial survivor data?
An indirect answer is that any tool is a poor tool when it is misused.
It is our opinion that, when properly used, the D-value is a very good
tool to communicate microbial survival data. Let us proceed to examine
some data where it is appropriate to present data using D-values followed
by some inappropriate uses of the D-value.

In Figure AE.3 are shown survivor curves where aliquots of the
same spore suspension have been inoculated onto similar planchets; these
planchets have in turn been divided into four lots; one lot was heated
at a relative humidity (RH) of 15%, the second lot was heated at an RH
of 35%, the third lot was heated at an RH of 55% and the fourth lot
heated at an RH of 75%. In each case, replicate numbers of planchets
were heated for several lengths of time after which the survivors were
recovered and enumerated. In Figure AE.3 are shown the data points,
the straight line survivor curves, and the D-values. In this example
the survivor data, for the different test RH conditions as a function of
heating time, form essentially straight lines when the logarithm of the
number of survivors is plotted as a function of temperature. Therefore
the D-value, which is a measure of the direction of this line, is an
ideal way of comparing the effect of RH on microbial survival. We can
convey to the reader the effect of RH simply by indicating that at 15% RH
the D-value is 121 hours, at 35% RH the D-value is 59.3 hours, at 55% RH
the D-value is 32.9 hours, and at 75% RH the D-value is 1.09 hours. This
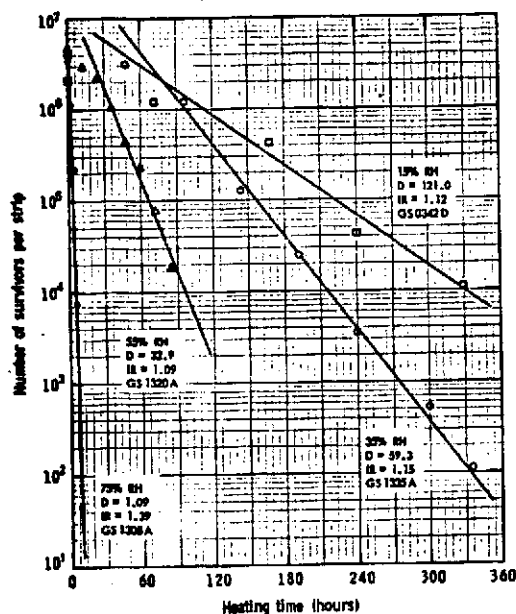is a good use of D-value to summarize microbial survivor data.

Figure AE.3: Survivor curves for
B. subtilis var. niger spores (AAOE)
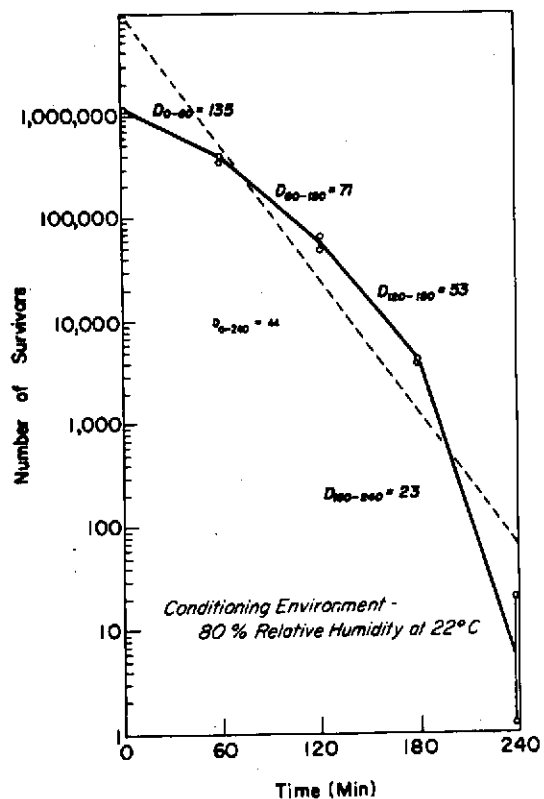heated at 90°C and at 15, 35, 55,
and 75% RH.

Figure AE.4: Survivor curve for
AAOE spores at 125°C, 80% RH, 22°C.

The D-value can be misused in many ways; in the area of data analysis
it is wrong to use the straight line D-value summary for curvilinear
survivor data.   In Figure AE.4 is shown the survivor data for Bacillus
subtilis spores AAOE heated at 125°C in a heat-block where the initial
humidity of the atmosphere surrounding the spores was 80% RH at 22°C.
The dotted line in Figure AE.4 is the least squares "best-fitting" line
that we would use if we were going to summarize these data with a single
straight line and calculate a D-value; the D-value of the dotted line is
44 minutes.   In Figure AE.4, we show the 2-point D-values for the initial
segment (0 to 6.0 min) D = 135 min; from 60 to 120 min D = 71 min; from
120 to 180 min D = 53 min; and from 180 to 240 min D = 23 min. We feel
that it is reasonable to calculate 2-point D-values and use these values
to discuss how the rate of destruction changes as heating time increases.
However we believe that to report a single D-value for all of these data
would be misleading to the reader, in the extreme!

The second area of abuse of the D-value is in using a D-value that was obtained over a limited microbial destruction range to construct a new survivor curve that extends considerably beyond the original range of measurement. One of the most flagrant misuses of the D-value may seem like ancient history to the younger reader but to those of us who lived through the episode it still seems like yesterday. We are referring to the inactivation of polio virus by Formalin by the Salk method and the use of this method of sterilization in the commercial manufacture of vaccine.[1]

Figure AE.5 shows the inactivation curve of polio viruses by Formalin from about 10^6 to 10 particles over a given time interval (solid line). The dotted line is the extrapolation of these experimental data to predict inactivation requirements for commercial quantities of vaccine. When one vaccine manufacturer proceeded to expose his virus particles for the production of commercial quantities of vaccine using times from the extrapolated curve, the entire health community was shocked because the vaccine, which according to theory was supposed to contain only killed viruses, must have contained a few live viruses. The use of this vaccine resulted in a fairly large number of cases of paralytic polio and about a dozen deaths.
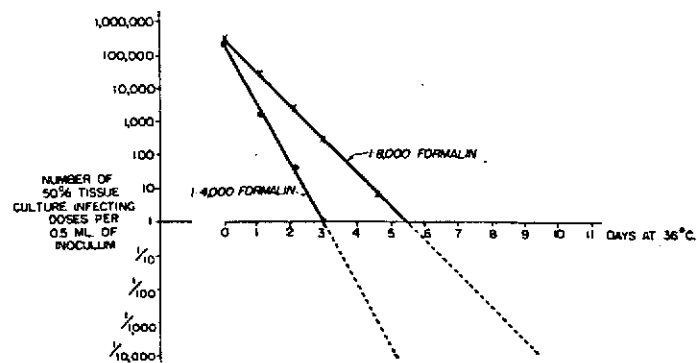
Rate of Destruction of Infectivity of Poliomyelitis Virus in Tissue Culture Fluid—Treated with Formalin at 36° C. and at pH 7.0

Figure AE.5: Inactivation curve of polio viruses (data from Salk et al.1954).

[1] Salk, J.E.; Krech, U.; Youngner, J.S.; Bennett, B.L.; Lewis, L.J.; and Blazeley, P.L. (1954): Formaldehyde treatment and safety testing of experimental poliomyelitis vaccines. Am. J. of Pub. Health, 44, No. 5, pp. 563-570.

This is sound testimony to the fact that the shape of a survivor curve is determined by the specific organisms that are under consideration under the particular test condition being used; furthermore we cannot say with any confidence at all that the shape of the survivor curve, if it is a straight line over the area in which we have tested, will continue to be a straight line into areas where we have not tested. Here again the D-value as a tool is not at fault. It is rather the hazardous misuse of the tool, and the individuals who misused it, who must take the responsibility.

Identifying the semi-log survivor curve

We have established that a good way of treating microbial survivor data is to plot these data on a semi-log graph; hopefully, the data will form a single straight line, or if not one straight line, the data will be well represented by two straight lines. We are now going to discuss further identification of straight lines used to represent survivor data on semi-log graphs.

To identify the straight line that represents the best estimate of the survival characteristics of a microbial culture requires the location of two points on the line or one point and the slope of the line. The D-value is a measure of the slope of the survivor line but we must have, in addition to the slope, the location of a point on that line if it is to be described. We have recently introduced the use of intercept ratios (IR)

$$IR = \log (Y_o/N_o)$$

along with the D-value so that the line is further identified.

If we proceed to summarize data that can be represented first by a single straight line and through modifications which, at last, are representable by two straight lines we will note that there are several variations even in this limited area. We have the following evolving variations (assume, in all cases, that we have at least six survival points in addition to N_o):

    1. A single straight line passing through N_o.

    2. Data points that, at all times greater than zero, form a straight line; the Y intercept is either significantly greater or smaller than the measured N_o.

3. Data points 2, 3, 4, 5, 6, ... or 3, 4, 5, 6, ... form a straight line. The N_O and data point I (and perhaps 2) do not fall on a straight line.

4. Data points N_O, I, 2, 3 form a straight line and data points 3, 4, 5, 6, -, -, - form a second straight line.

The treatment of the above types of data is as follows:

When the data points form a straight line that either does or does not pass through N_O, the data are adequately identified if the D-value and IR are reported. It is normal practice in our data analysis to calculate a D-value and IR for data points only (N_O is excluded). This type of survivor data is shown in Figure AE.2.

The survivor curve shown in Figure AE.6 is of the shape that is often obtained when very dry spores on stainless steel planchets are subjected to dry heat. In this situation we have an initial very rapid die-off of 90-95% of the spores followed by a straight line logarithmic destruction at a slower rate. The D-value and intercept ratio allow us to portray this type of data; it is important that the N_O value not be included in the regression analysis.
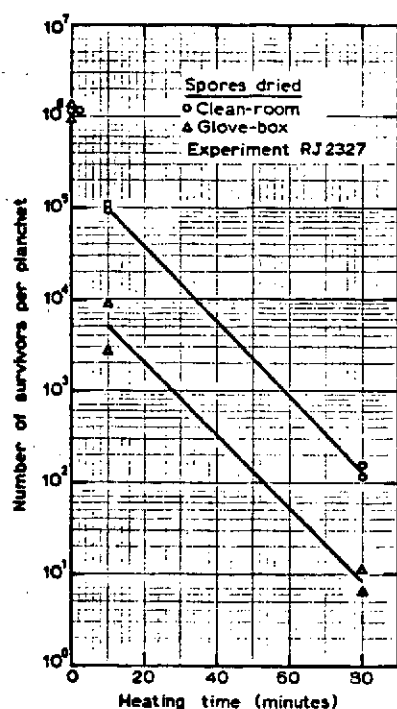

Figure AE.6: Survivor curves for spores of B. subtilis var. niger on stainless steel planchets subjected to dry heat at 110°C.
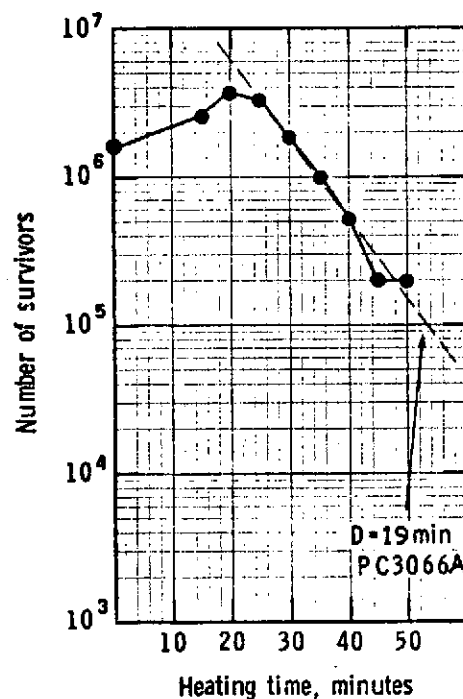

Figure AE.7: Survivor curve for spores PCFF on paper carriers in aluminum foil envelopes 2.5 X 4.5 inches heated at 115°C (PC3066A).

Figure AE.7 is a survivor curve where we have an initial static period followed by logarithmic destruction. Here, we would suggest that the D-value be related to the straight line portion as indicated, and that the intercept ratio be used to indicate the fact that we have an initial period where the destruction rate is low.

When the survivor data for all data points except the first or first and second (and except N_o) form a straight line, then we suggest that the data points that fall on a straight line be so represented and that the straight line be identified by the IR and the D-value. The time range over which the data points form a straight line should be represented as D_{125} = 19 min (20, 50 min).

In Figure AE.8 is shown a survivor curve for <u>Bacillus</u> <u>subtilis</u> var. <u>niger</u> spores (AAOE) heated on stainless steel planchets at 75% RH (GS1308A). In this test there were five data points: N_o, 2, 4, 6, and 8 hours. We analyzed the data first using the data from the four heating times. We then reanalyzed the data using only values at 4, 6, and 8 hours. The results are shown in Figure AE.8 and are tabulated below:

data used	D-value	confidence limits	IR
2, 4, 6, 8 hrs	1.36 hrs	1.17-1.63	1.20
4, 6, 8 hrs	1.09 hrs	.911-1.34	1.39

When there is a clear indication of two straight lines, then the IR and D-value of the first straight line and the D-value of the second straight line should be given. The D-values should include the time range over which this line extends. In Figure AE.9 is shown the survivor curve for the natural contaminants in Dinkytown soil in ethanol (SM1292B). In this test we have calculated the straight line for the first three data points (excluding N_o) and then for data points three through six. The D_1-value was 0.35 hours (0.25 to 1.0 hours); IR = 0.5; D_2-value was 8 hours (1.0 to 8.0 hours).
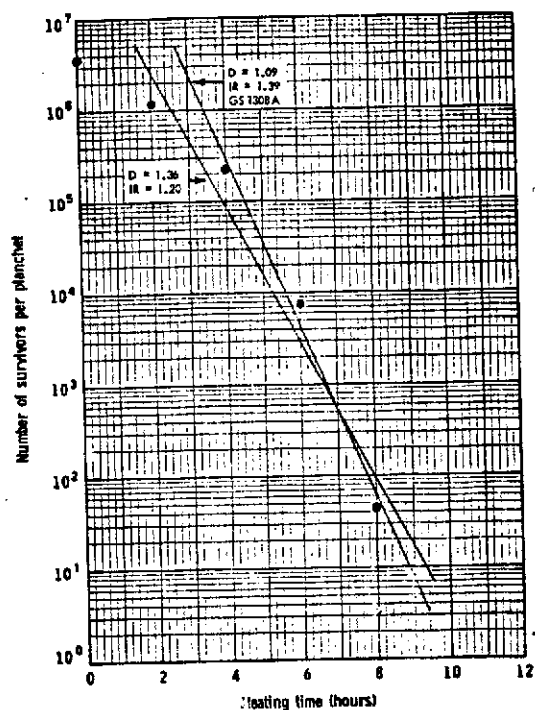
Figure AE.8: Survivor data for Bacillus subtilis
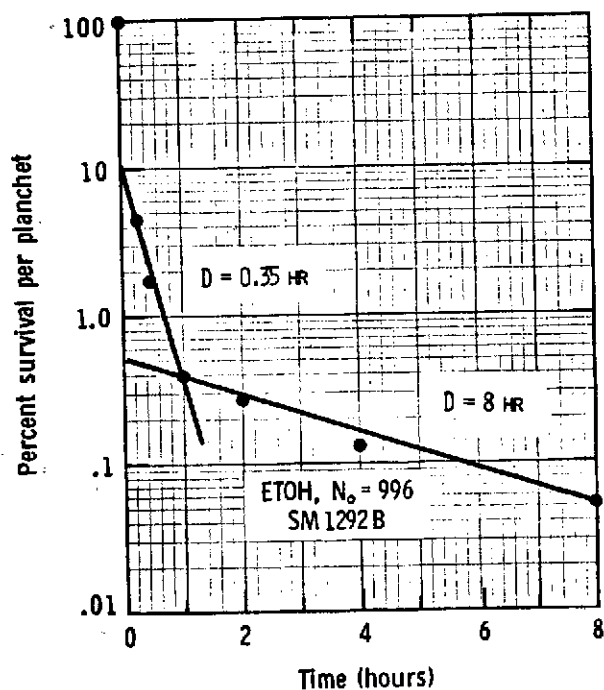var. niger spores analyzed using all points and
omitting the two hour data point.

Figure AE.9: Dry heat survival characteristics
of organisms in Dinkytown soil suspended in
ethanol and heated at 125°C.

Using the computer to aid in data analysis

We believe that it is very important for the experimenter personally to observe the pattern formed when the survival points are plotted on the survivor curve. To this end, we have developed a procedure in our laboratory for handling our data whereby the computer plotter produces a graph of the data. (The logarithm of the number of colony-forming units is plotted versus time.) Data values and confidence intervals are indicated on the graph; the mean-value points are connected by straight lines. The experimenter can visually note the shape of the survivor curve on the graph. A computer plot is shown in Figure AE.10.

The next stage in our data handling system will be to instruct the computer to inspect these data and determine if the data can best be represented by one or two straight lines or a combination of lines and curves. It is only after the data have been inspected and a decision made regarding the pattern of the data points that parameters can be evaluated and statistical calculations can be carried out.

Replication of experiments

It now follows that if the microorganisms that we are studying are sensitive to environmental conditions and the variations between such environmental conditions are smaller than the variation in the environmental conditions of our control systems, then replication is a vital part of our experimental procedures. With replication we can reduce the mean microbial response due to uncontrolled environmental fluctuation. As a minimum, all experiments must be duplicated with the duplicate experiment being carried out on a different day from the first experiment. It is probable that three replications carried out on three different days would be more desirable when time and resources permit.

A starting point in all studies involving microorganisms must be the acceptance of the proposition that the individual microorganism, or the microbiological population as a whole, responds to the stress condition in a reproducible manner; that is, if the same microorganisms are repeatedly subjected to the same stress conditions the same results will be obtained.

A microorganism must be viewed as a biological system where changes in the chemical characteristics or the physical environment or both produce an effect over time. This effect is measured in a simplistic manner by observing whether or not the microorganism is able to produce a colony on solid media, or sufficiently large numbers to produce turbidity in
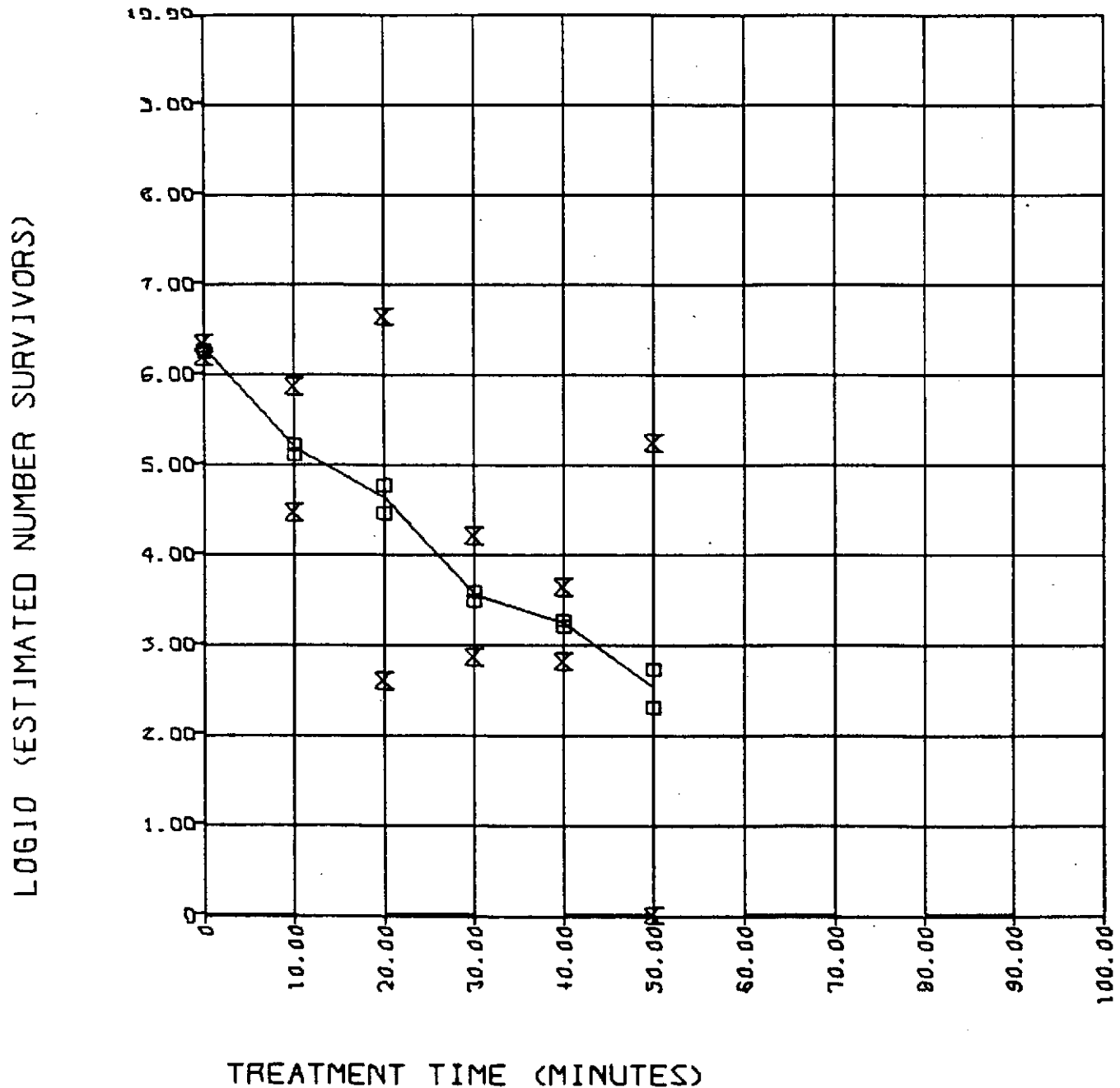
99

Figure AE.10: Computer prepared graph of experimental results: the line passes through sample means. Both data points (□) and confidence limits (X) are shown.

liquid media, under favorable growth conditions. Changes in the destruction pattern of the microorganism as a function of environmental conditions should not be interpreted as a change in the organism but should be interpreted as a change in the environment.

When a homogeneous culture of microorganisms is subjected to assumed identical environmental stress conditions and widely varying results are obtained, this variation is often attributed to the microorganisms; but fluctuations in environmental conditions are often responsible for the variations. The microbial cell is extremely sensitive and will respond predictably to changes in environmental conditions in such a way as to integrate the effect of chemical and physical changes acting over time. Meaningful results cannot be obtained where fluctuations in the environment produce responses in the microorganisms comparable to responses produced and measured by the experiment itself. It is vital that the environmental variables be controlled, and one of the "controls" should be replication.

Observing experimental data

There are some basic ground-rules that are necessary and need to be followed in microbiological sterilization studies. When we subject microorganisms to lethal agents, we must assume that we are operating under conditions that will preclude reproduction that would produce new viable cells. Certainly, during heat destruction tests when micro-organisms are dry and are exposed to dry heat, radiation, or chemical sterilants, neither growth nor reproduction will take place. This assumption sets the stage for a postulate put forth by Schmidt (1950)[2] stating that, in the analysis of thermal destruction data, if after a specified heat treatment a microorganism survived, this organism would survive in a shorter heating period (a less amount of stress). He also feels that if after a specified heating time a specific percentage of microorganisms is inactivated or destroyed, that at least this percentage of microorganisms will be destroyed at any longer time.

If a microorganism in an aliquot of a homogeneous microbial spore suspension survives after ten minutes at 125°C, for example, at 125°C there will be survivors at all times shorter than ten minutes. If all

[2]Schmidt, Clarence F. (1950): A procedure for the interpretation of thermal death time data; Memorandum to the N.C.A. processing subcommittee on foods in metal containers; National Canners Association; Washington, D.C.

microorganisms are killed after 20 minutes at 125°C, then if the aliquot is heated for times longer than 20 minutes at 125°C, they would also be killed.

Conclusions

Review of the problems associated with analysis and interpretation of microbial survival data indicates a need for a set of criteria that can be used in the treatment and analysis of these data. The criteria we are proposing require that a judgment be made by the scientist to classify his data after which the statistical analysis will be carried out. (Perhaps in the future computer programs can be developed to make these judgments.) The procedure we propose is based on experience gained from working with a large quantity of microbiological survivor curve data and in discussions with the scientists working in this area. We believe that it is important that we begin to use the knowledge that we have been gathering regarding survivor curve shapes to further our knowledge of microbial behavior during sterilization processes.

Our recommendations are listed below:

1. Duplicate or triplicate tests, each on a different day, should be carried out at each test condition. The raw data is the starting point in the analysis. We must be able to have confidence in the results.

2. The data should be plotted on a survivor curve graph. The semi-log type survivor data graph is recommended where the number of survivors is plotted directly on a log scale (or the logarithm of the number of survivors is plotted on an arithmetic scale) as a function of the heating time or intensity of the chemical stress.

3. Inspection of the survivor data on the semi-log graph to determine the representation scheme to be followed.

4. The survivor data should be subjected to statistical analysis that will yield (a) confidence intervals around the replicate data points, (b) 2-point D-values, and (c) straight lines for the data (if justified). When D-values are reported, the time interval over which D-values are calculated should be given.

5. The intercept ratio (IR) which is the logarithm of the ratio of the y-intercept (Y_o) of the least-squares fit line to the initial number (N_o) of organisms, should be calculated when one or two straight lines describe the data. If there are two straight lines, an IR value is calculated only for the first straight line. A survivor curve is well

described if the N_o, IR, and D-values are reported.

6. If the data seem to form a single straight line either including or excluding N_o, then carry out the regression analysis of the data either including or excluding N_o as the situation warrants. Report the D and IR values.

7. If the data seem to form a single straight line excluding N_o and the first portion of the heating interval, carry out a regression analysis of the data excluding N_o and those "first portion" points from the analysis, provided that a sufficient number of data points remain for analysis. Report the IR value and D-value and give the time over which D-value was calculated.

8. If the data form two straight lines either including or excluding N_o then carry out regression analysis of data for each line appropriately. Report the IR and D-value for the first line along with its interval of calculation, and also the D-value for the second along with its time limits.